

1992

Pathology and diagnosis of chlamydial abortion in ewes

Thomas Paul Sanderson
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Pathology and diagnosis of chlamydial abortion in ewes

Sanderson, Thomas Paul, Ph.D.

Iowa State University, 1992

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Ann Arbor, MI 48106**

Pathology and diagnosis of chlamydial abortion in ewes

by

Thomas Paul Sanderson

A Dissertation Submitted to the Graduate

Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Veterinary Pathology

Major: Veterinary Pathology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

**Iowa State University
Ames, Iowa**

1992

TABLE OF CONTENTS

1.	INTRODUCTION	1
	Explanation of Dissertation Format	3
2.	LITERATURE REVIEW	4
	Chlamydiae	4
	Ovine Chlamydial Abortion (Enzootic Abortion of Ewes)	14
	Other Infectious Causes of Ovine Abortion	27
	Non-infectious Causes of Abortion	37
3.	GROSS AND MICROSCOPIC PATHOLOGY OF OVINE FETUSES INFECTED WITH <i>CHLAMYDIA PSITTACI</i>	38
	Introduction	38
	Materials and Methods	39
	Results	44
	Discussion	85
4.	IDENTIFICATION OF <i>CHLAMYDIA PSITTACI</i> IN FORMALIN- FIXED, PARAFFIN-EMBEDDED OVINE FETAL TISSUES WITH AN AVIDIN-BIOTIN-PEROXIDASE COMPLEX (ABC) IMMUNOENZYMATIC TECHNIQUE	91
	Introduction	91
	Materials and Methods	92
	Results	97
	Discussion	113

5.	AN ULTRASTRUCTURAL STUDY OF CHLAMYDIAL PLACENTITIS	116
	Introduction	116
	Materials and Methods	117
	Results	119
	Discussion	155
6.	EVALUATION OF AN ENZYME IMMUNOASSAY FOR DETECTION OF <i>CHLAMYDIA PSITTACI</i> IN VAGINAL SECRETIONS, PLACENTAS, AND FETAL TISSUES FROM ABORTING EWES	159
	Summary	159
	Introduction	160
	Materials and Methods	161
	Results	166
	Discussion	172
	References	174
7.	EVALUATION OF A RAPID SOLID PHASE IMMUNOASSAY FOR DETECTION OF OVINE <i>CHLAMYDIA PSITTACI</i>	180
	Summary	180
	Introduction	180
	Materials and Methods	181
	Results	184

	Discussion	185
	References	187
8.	AN INDIRECT MICROIMMUNOFLUORESCENCE TEST FOR DETECTION OF CHLAMYDIAE-SPECIFIC ANTIBODIES IN OVINE FETAL FLUIDS	189
	Summary	189
	Introduction	190
	Materials and Methods	191
	Results	198
	Discussion	206
	References	208
9.	GENERAL DISCUSSION AND SUMMARY	214
10.	LITERATURE CITED	219

1. INTRODUCTION

Chlamydiae are a genetically diverse group of gram-negative obligate intracellular bacteria that cause economically important diseases in humans, domestic mammals, and birds. There are 3 species; *C. trachomatis* and *C. pneumoniae*, are primarily human pathogens, and *C. psittaci* is a pathogen of both humans and animals. Most avian species and many domestic and feral mammals have been shown to be natural hosts for *C. psittaci*.^{265,316} Infections of various animals with mammalian strains of *C. psittaci* have been associated with abortion and reproductive failure,^{2,95,285,323,337,339,341,347,392} polyarthrititis and polyserositis,^{166,285,309,324,337,338,338,341} pneumonia,^{46,254,337,338,341} enteritis,^{96-99,116,276,338,392} meningoencephalitis,^{154,172,172,323,337,338,341} and conjunctivitis and ocular disease.^{46,115,166,167,172,323,337,338,341,386}

Ovine chlamydial abortion or enzootic abortion of ewes is a major cause of reproductive loss in sheep. Chlamydial abortion has been identified worldwide in most major sheep producing areas where often it is the most commonly diagnosed cause of ovine abortion.^{6,7,71,165,203,224,241,248,289,322,323,339} Infection of susceptible pregnant sheep with *C. psittaci* often results in a severe suppurative placentitis.

Confirming a diagnosis of chlamydial abortion can be challenging. A tentative diagnosis often is based upon the flock history and the presence of characteristic placental lesions. These fastidious intracellular organisms are often difficult to isolate, especially under field conditions. They are readily inactivated in the course of intrauterine events that lead to fetal death.^{214,337,339,345} Contamination by other bacteria and unfavorable transport conditions

further reduce the viability of chlamydiae.³³¹ There usually are no diagnostic lesions in ovine fetuses infected with chlamydiae. In contrast to the situation in humans, few direct (non-culture) diagnostic tests have been developed or evaluated for diagnosis of chlamydial infections in sheep. No doubt, better diagnostic procedures for chlamydial infections in sheep are needed.

The objectives of this study were: 1) evaluate the gross, microscopic, and ultrastructural changes in ovine placentas and fetal tissues induced by infection with *C. psittaci*, 2) to develop an immunohistochemical technique for detection of chlamydial antigen in formalin-fixed paraffin embedded ovine fetal tissues, 3) develop an indirect microimmunofluorescence serologic procedure for detection of chlamydial antibodies in ovine fetal fluids/serum, and 4) determine if direct (non-isolation) diagnostic tests designed to detect *C. trachomatis* infections in human specimens, were useful for diagnosis of chlamydial infections in sheep.

The National Animal Disease Center - National Veterinary Services Laboratories' Animal Care and Use Committee reviewed and approved this study and determined that the animals were treated humanely in all procedures involved in these studies.

The Ph.D candidate, Thomas P. Sanderson, was the principal investigator for each study.

Explanation of Dissertation Format

This dissertation contains three manuscripts written as chapters (Chapters 6,7,8) that have been published in or submitted to the Journal of Veterinary Diagnostic Investigation, a refereed scientific journal. Each manuscript, although treated as chapters, has references at the end. A literature review precedes chapters on gross and microscopic pathology, immunohistochemical studies, and ultrastructural changes. A general summary and literature cited in the introduction, literature review, and chapters on gross and microscopic pathology, immunohistochemistry, and ultrastructure follows the last chapter.

2. LITERATURE REVIEW

Chlamydiae

Chlamydial Nomenclature and Taxonomy

Chlamydiae are a genetically diverse group of gram-negative obligate intracellular bacteria with similarities in morphology, intracellular development, and antigenic properties. There are 3 species; *C. trachomatis* and *C. pneumoniae* are primarily human pathogens, and *C. psittaci* is a pathogen of both humans and animals.

Historically, chlamydiae have had many names including *Bedsonia* microorganisms, *Miyagawanella* agents and psittacosis-lymphogranuloma venereum-trachoma organisms.³⁵⁰ When first identified in conjunctival scrapings they were thought to be protozoan due to the size of their intracellular inclusions.³¹² Later, chlamydiae were considered to be large viruses because of their obligate intracellular growth in eukaryotic cells. However, it then became obvious that chlamydiae were bacteria because they possess a cell envelope similar to gram-negative organisms, contain both DNA and RNA, divide by binary fission, possess prokaryotic ribosomes, synthesize proteins, nucleic acids, and lipids, and are susceptible to antibiotics.^{260,393} Chlamydiae also were considered among the *Rickettsia*, but have been differentiated from them as well.^{312,384} In contrast to rickettsia, chlamydiae lack a system for electron transport, have no cytochromes, cannot synthesize ATP and GTP, and do not preferentially use glutamate.³⁸⁴

C. trachomatis is an important cause of keratoconjunctivitis (trachoma) and genital infections in humans.^{136,311,316} Trachoma is a disease of poverty and is the world's leading

preventable cause of blindness.^{227,311} In western societies, *C. trachomatis* is the most commonly sexually transmitted pathogen.^{40,199,272,311,316,317,361} Ascending genital infection is common. Acute pelvic inflammatory disease,^{227,311} infertility, and ectopic pregnancy are common sequelae of chlamydial genital infections.^{316,343} Other diseases associated with infection with *C. trachomatis* include urethritis,^{302,311,316} arthritis,²⁰⁹ neonatal conjunctivitis and pneumonia,^{302,311} inclusion conjunctivitis in adults,^{311,313,316} and lymphogranuloma venereum.^{311,313,316,338} *C. pneumoniae* is an important cause of upper respiratory disease and pneumonia.^{136,138,284} Most infections are mild or asymptomatic, but occasionally severe pneumonia and death have occurred.^{36,171}

In contrast to *C. trachomatis* and *C. pneumoniae*, *C. psittaci* are less host and tissue specific. Most avian species and many domestic and feral mammals have been shown to be natural hosts for *C. psittaci*.^{265,316} Avian chlamydiosis affects both domesticated and wild birds.^{21,265,387} The organism is shed in the feces, contaminates the environment, and is spread by aerosol.^{341,387} The respiratory tract is the primary route of infection. Chlamydial infection of birds may result in pneumonia, airsacculitis, tracheitis, hepatitis, myocarditis, splenitis, nephritis, orchitis, enteritis and encephalitis.^{11,21,134,338,341,387}

Infections of various animals with mammalian strains of *C. psittaci* have been associated with several diseases including abortion and reproductive failure,^{2,95,285,323,337,339,341,347,392} polyarthritis and polyserositis,^{166,285,309,324,337,338,338,341} pneumonia,^{338,341,46,254} enteritis,^{96-99,116,276,338,392} meningoencephalitis,^{154,172,172,323,337,338,341,341} and conjunctivitis and ocular disease.^{46,115,166,167,172,323,337,338,341,341,386}

Chlamydiae have been assigned to the order *Chlamydiales*, which is composed of one family, *Chlamydiaceae*, with a single genus, *Chlamydia*.²⁶⁰ Until recently all members of this genus were classified under the species of *Chlamydia trachomatis* and *Chlamydia psittaci*.^{136,138,257,260} These two species were differentiated on the basis of sulfonamide sensitivity and presence of iodine-staining inclusions: *C. psittaci* is resistant and *C. trachomatis* is susceptible to sulfonamides; *C. trachomatis* inclusions stain with iodine while *C. psittaci* inclusions do not. A third species, *C. pneumoniae*, has recently been identified.^{63,136-138,284} It differs from the other species ultrastructurally, antigenically, in DNA composition and in attachment and uptake.^{63,137,138,203,313,375} Several unique serotypes of *C. pneumoniae* have now been identified.³⁶

C. trachomatis has been further subdivided into biovars mouse pneumonitis, trachoma, and lymphogranuloma venereum (LGV); each biovar can be identified serologically. The mouse pneumonitis agent is antigenically distinct from the more closely related trachoma and LGV biovars. DNA homology within the trachoma and LGV biovars is almost complete.³¹² The mouse biovar is less related (30-60% homology) and has less than 10% homology with *C. psittaci*. The trachoma biovar contains 15 serovars (A, B, Ba, C, Da, D, E, Ea, F-H, I, Ia, J, K), and the LGV biovar contains 4 serovars (L1, L2, L2a, L3).^{352,376} Serovars have been differentiated antigenically with monoclonal antibodies.^{352,376} Antigenic determinants of serovar specificity reside in the major outer membrane protein (MOMP). The molecular basis of antigenic diversity and interrelationships among serovars has been confirmed by sequence analysis of the MOMP genes.^{62,86,394}

Because *C. psittaci* is very heterogenous, classification of the diverse strains has been challenging. Subspecies differentiation has been based upon inclusion morphology,³³⁰ plaque neutralization,³¹⁴ serology,³¹⁵ and immunoblot analysis.³⁵⁶ Mammalian strains of *C. psittaci* have been subgrouped into nine immunotypes using an indirect immunofluorescence technique.²⁸¹ Of particular importance in sheep are immunotype 1, causing abortion; and immunotype 2, associated with arthritis and conjunctivitis. Immunotype 9 has been isolated from sheep feces but has not been associated with disease.²⁸¹ Recently, indirect microimmunofluorescence (IMIF) techniques in combination with restriction endonuclease analyses and serovar specific monoclonal antibodies have proven useful for classification of *C. psittaci*.^{9,10,233,356}

Morphology and Structure

Chlamydiae are obligate intracytoplasmic bacterial parasites with a complicated unique life cycle. Depending upon the stage of the developmental cycle, there are several morphologic forms. Chlamydial forms can be differentiated according to morphology, chronological appearance during multiplication, function, strength against mechanical agitation, cell wall permeability, and chemical composition of cell envelopes.^{230,341}

Elementary bodies (EB), the extracellular infectious forms, are metabolically inert, nonmotile, and dense, roughly spherical particles that range from 0.2 to 0.3 μm in diameter. They are relatively resistant to the extracellular environment and permit the infection to spread from one cell (or one host) to another.^{312,316,338} The EB have an outer trilaminar membrane that surrounds a narrow less distinct trilaminar plasma membrane.³⁴¹ An electron-

dense nucleoid is eccentrically located and in contact with the plasma membrane. The remainder of the EB is less dense than the nucleoid and is composed of closely-packed ribosomes and moderately dense amorphous material.^{230,231} Reorganization from infectious EB to the noninfectious reticulate bodies (RB) proceeds through dispersing forms.³⁴⁴ They contain a granular matrix that is less dense than the nucleoid of EB but more dense than RB. The RB is the intracellular, metabolically-active vegetative form that divides by binary fission. RB range from 0.6 to 1.3 μm in diameter, are osmotically fragile, and are surrounded by an inner and outer trilaminar membrane.^{230,312,316,338} Internal structure of RB consists of numerous ribosomes and interspersed fine reticulated fibers. Smaller reticulate bodies are transformed, through condensing forms (intermediate bodies-IB) into the infectious elementary bodies.

In contrast to other gram-negative bacteria, chlamydiae lack peptidoglycan between the outer and inner membranes.^{23,126} The outer membrane is composed of hexagonal subunits of the major outer membrane protein (MOMP) that connect to transmembrane channels.^{216,231} Ten to thirty hemispheric projections that are hexagonally arranged at a center to center spacing of 50 nm are present on the surface of EB.^{230,258} Each projection emerges from a rosette-like pore that is approximately 30 nm in diameter and is anchored in the cytoplasmic membrane.³⁹³ These surface projections may be involved in uptake of nutrients, however, they apparently play no role in attachment of chlamydia to host cells.^{139,230}

Genus specific lipopolysaccharide (LPS) and the MOMP are major components of the chlamydial cell wall.⁵⁸ The MOMP is a transmembrane protein that is linked by disulfide

bonds to other cysteine-rich membrane proteins to maintain structural rigidity. Exposure of the EB to reducing conditions in phagosomes breaks these disulfide bonds, making the cell wall less rigid and allows the structural changes that occur when the EB changes into an RB.^{160,264} Reduction also results in porin formation³² whereby the outer membrane becomes permeable to nucleotides and amino acids required for RB metabolism.

The MOMP contains serovar-, subspecies-, species- and genus immuno-domains.^{6,10,59,74,202,251,334,369,397} Although the MOMP is antigenic in human and animal infections, the role of MOMP-specific antibodies either in protective immunity or for diagnosis is unclear.^{28,31,251} Polyclonal and monoclonal antibodies to MOMP neutralize chlamydial infectivity in cell culture.^{57,59} Recent studies have demonstrated that a single dose of a MOMP-enriched subcellular preparation of detergent extracted EB protected ewes against a subcutaneous challenge with a live homologous strain of *C. psittaci* at 70 days gestation.³⁵⁹ Apparently different antibodies may neutralize chlamydial infectivity at different stages of the developmental cycle.²⁵⁸

Chlamydial LPS strongly resembles the lipopolysaccharide (LPS) of other gram-negative bacteria in its location in the outer membrane of the chlamydial envelope, chemical structure, and biological activity.^{41,57,269,270} It (LPS) contains at least three antigenic domains, two of which are shared with the LPS of certain other gram-negative organisms including *Salmonella* rough mutants and *Acinetobacter calcoaceticus*, and a third domain that is specific for chlamydiae.^{57,270} Extractable chlamydial LPS is the antigen detected with the standard complement-fixation (CFT) serologic test.³³⁷ In addition, monoclonal antibodies

and polyvalent antisera to the LPS have been used in enzyme immunoassay systems to detect chlamydial antigen in various clinical specimens.

Chlamydiae contain three penicillin-binding proteins in the cytoplasmic membrane, but lack peptidoglycan. Although peptidoglycan is absent, chlamydiae are inhibited by penicillin and lysozyme.¹²⁶ A satisfactory explanation for the anti-chlamydial activities of penicillin and lysozyme has not been determined.

Developmental Cycle

The developmental cycle of chlamydiae consists of 5 major phases: 1. attachment and uptake of the EB; 2. transition of the metabolically inert EB into the metabolically active RB; 3. growth and division of the RB; 4. maturation of noninfectious RB into infectious EB; and, 5. release of EB from the host cell.^{209,256,312,316,337,341,393}

The intracellular developmental cycle begins with attachment of the EB to microvilli at the apical surfaces of susceptible cells. The entry-promoting property of the EB is an intrinsic property of the cell wall and may involve a specific receptor-ligand interaction.^{259,368} Competition assays of adherent EB on host cells have demonstrated that heat labile and trypsin-sensitive chlamydial moieties are involved in adherence.^{259,368} Solubilized chlamydial membrane proteins including trypsin-resistant heparin binding proteins and EB-specific proteins (18 and 31 Kd *C. trachomatis*, 16 and 29 Kd *C. psittaci*) have been shown to bind to susceptible host cell membranes.^{135,146} These proteins are present on EB but not on RB. Monoclonal antibodies and antiserum prepared against these binding proteins inhibited the infectivity for *C. trachomatis* for HeLa cells.^{187,188} The MOMP also may function as a

chlamydial adhesin.³⁵¹ Histone H1 was identified as a chlamydial-binding host cell protein in HeLa cell membrane fractions.³⁷³ Recently, it was proposed that albumin bound to the EB surface acting via albumin-binding surface receptors on host cells may complement other attachment mechanisms.³³⁵ Seemingly, chlamydiae may have several methods for binding to host cells.

Once attached, the EB is rapidly internalized. It is unclear whether EB enter host cells by means of microfilament-dependent phagocytosis or receptor-mediated endocytosis. Most of the evidence favors endocytosis via a clathrin-dependent process.^{257,258} Chlamydiae enter the cell within an endosome and remain there throughout the entire life cycle.^{209,256,312,316,337,341,393} They evade killing by inhibiting endosome-lysosome fusion, possibly in the same manner as other intracellular pathogens such as *Toxoplasma gondii*, *Legionella pneumophila*, and *Mycobacterium tuberculosis*.^{73,129,182,217} Inhibition of endosome-lysosome fusion is limited to endosomes containing intact EB.¹¹¹ Components of the cell wall that can be inactivated by heating or by combination with antibody are necessary for inhibition of endosome-lysosome fusion.^{111,112,129,351} These protective elements are either absent from the RB wall or are present in an inactive form. Differences in endosome membrane proteins or some membrane marker may prevent fusion with lysosomes.^{129,179,258} Endosomal membranes surrounding viable *C. psittaci* EB contain a 70 Kd protein that is not present in membranes of phagosomes containing heat-inactivated EB.³⁹⁵ A unique 25 Kd protein and reduced amounts of 36 and 38 Kd proteins are present in phagosomes containing heat-inactivated *C. psittaci* EB.³⁹⁵ Recent studies have demonstrated that inhibition of endosomal acidification may be the mechanism

by which chlamydiae inhibit fusion with lysosomes.³⁶⁰ Apparently, molecules on the outer membrane of the EB are responsible for effecting the block of endosomal acidification and this block is initiated when the EB first attaches to the cell.³⁶⁰

Upon entering the host cell, the chlamydial EB organizes into a reticulate body (RB).^{312,316,338,378} The mechanisms controlling EB-RB reorganization are poorly understood.^{121,372} A series of promoters that are sequentially recognized by an RNA polymerase modified by activating proteins may regulate the developmental cycle.²⁸² Protein synthesis and reduction of the disulfide-bond-cross-linked MOMP to its monomeric form are the first biochemical events detected in chlamydiae after uptake by host cells and is soon followed by synthesis of DNA and RNA.^{18,159,258,264,312,316,338,341} Once started, reorganization proceeds rapidly and by 8-12 hours after infection, almost pure populations of RB are present.²⁵⁸ Under optimum conditions multiplication by binary fission occurs rapidly. Because RB lack enzymes required for the net generation of energy, they utilize host cell ATP to generate the proton motive force necessary to transport essential nutrients across the cytoplasmic membrane.^{316,378,393} Chlamydial development is kept in step with the metabolic health of the host cell.^{156,393} Presumably, when nutrient supplies fall below critical levels, chlamydial development stops and RB reorganize to form infectious EB.^{54,56,156,316,378} There is no sharply defined end to the developmental cycle. Generally, the developmental cycle of *C. psittaci* is about 48 hours and that of *C. trachomatis* is slightly longer.

Early stages of the developmental cycle usually result in minimal degeneration of the host cell. However, as the cycle progresses, the expanding inclusion often occupies most of

the cytoplasm and displaces organelles. About 20-30 hours after infection cellular organelles show progressive degenerative changes including swelling, loss of ribosomes, dilation and vesiculation of the endoplasmic reticulum, loss of microvilli, and nuclear displacement and swelling or pyknosis.⁹⁷⁻⁹⁹ Release of host cell lysosomal enzymes results in breakdown of the inclusion membrane and cell lysis.^{64,365,366} Exocytosis of chlamydiae without cell death has been demonstrated for some strains of *C. trachomatis*.³⁶⁵

Metabolism

Chlamydiae lack the biochemical pathways for production of adenosine-triphosphate (ATP).^{312,316,338,381} Host cell ATP is utilized for phosphorylation of glucose to glucose-6-phosphate by hexokinase and to generate the proton motive force necessary to transport essential nutrients (i.e. amino acids) across the cytoplasmic membrane.³⁷⁸ Reticulate bodies utilize mitochondrial ATP via a chlamydial ATP-adenosine diphosphosphatase (ADP) translocase and metabolize it via a specific RB ATPase.¹⁵⁸ It has been suggested that the ATP-ADP translocase is part of a complicated network of nucleotide transport mechanisms that allow chlamydiae to use a number of different host generated energy-rich compounds both as substrates and as energy sources.¹⁵⁷ A GTP-GDP translocation system also has been identified in some strains of *C. psittaci* and *C. trachomatis*.¹⁵⁵

The nutritional requirements of chlamydiae are not highly dependent upon the host cell, but vary considerably between strains.^{7,72} Amino acids that chlamydiae incorporate into protein are either synthesized *de novo* or obtained from amino acid pools of the host.⁷ The concentration of amino acids within the cell is crucial. Amino acid antagonism may regulate

the growth of chlamydiae, probably by inhibiting the transport of required amino acids into the endosome.⁷² For example, gamma interferon inhibits chlamydial replication by decreasing levels of tryptophan which results in a relative increase in the antagonist which is phenylalanine.^{54-56,72}

Ovine Chlamydial Abortion (Enzootic Abortion of Ewes)

Ovine chlamydial abortion or enzootic abortion of ewes (EAE) is a major cause of infectious reproductive loss in sheep. Greig first described enzootic abortion in ewes in Scotland in 1936.¹⁴⁰ It was not until 1950 that Stamp and co-workers identified *Chlamydia psittaci* (*ovis*) as the cause of this disease.^{332,333} Ovine chlamydial abortion was first reported in North America in 1958 in Montana and subsequently was identified in sheep in Idaho, California, and Oregon.^{124,278} Chlamydial abortion has now been identified in most major sheep producing areas in the U.S., Canada, and Australia.^{3,4,68,161,198,219,237,245,286,320,321,337} It is the most frequently diagnosed cause of ovine abortion in the United Kingdom and Europe.^{2,4,4,43,50,338,341} Infection with *Chlamydia psittaci* has also been associated with reproductive failure in cattle,^{30,95,172,222,273,280,282,285,298,321,323,337,341,345} goats,^{19,44,115,144,303,323,337,341} horses,⁹⁵ pigs,^{285,323,392} and other domestic animals.^{27,174,175,385}

Chlamydial infection of susceptible pregnant ewes may result in abortion, stillbirths or delivery of small weak lambs.^{4,219,237,268,323,337,341} Some infected ewes give birth to normal live lambs. It is common to have one dead lamb and one or more weak or healthy lambs produced by the same ewe. Lambs are usually aborted in the last month of gestation, but abortions may occur as early as the 100th day of gestation.³⁴¹ The majority of lambs aborted

in late gestation are relatively fresh (minimal *in utero* autolytic changes), although mummified fetuses are encountered occasionally. Behavioral changes and abundant yellow-brown vaginal discharge may be present shortly before the abortion.^{1,329}

On initial exposure the incidence of abortion may be as high as 30% of the flock.^{1,2,4} Once the infection is established in the flock, abortions in 1-5% of ewes annually are common.^{323,341} Ewes of all ages may abort, but young ewes are most susceptible. Fertility and subsequent pregnancies usually are not affected.^{323,337}

Epidemiology

The epidemiology of chlamydial abortions in sheep is not completely understood. Inapparent intestinal infections with chlamydiae are common and the epidemiologic significance of these intestinal strains is uncertain.^{255,341} Serologic and immunoblotting studies have demonstrated that intestinal and abortion strains of *C. psittaci* are antigenically similar.^{141,304,330} Additionally, there is a high degree of antigenic conservation among abortion isolates and heterogeneity among the intestinal isolates.¹⁴¹ Speers and Storz demonstrated that fecal strains of chlamydiae, when administered parenterally to pregnant cattle could invade the placenta and fetus and cause abortion, but only with extremely high (superinfective) doses.^{285,337,341} Other studies have shown that abortion and intestinal isolates of chlamydiae differ in their invasiveness in pregnant ewes and mice.^{300,304} With few exceptions the abortion strains invaded and readily colonized the placenta and fetus while the intestinal strains did not. Attempts to distinguish abortion and intestinal isolates using plaque reduction, inclusion morphology, and microimmunofluorescence procedures have

produced conflicting results.^{109,141,177,281} Differences in invasiveness have been linked to variations in polypeptide profiles of purified abortion and intestinal isolates.^{52,176,233,234} Variations in the virulence of abortion strains for sheep and mice also have been reported, but no clear differences have been detected in culture or with DNA restriction analysis.^{5,13,176,304} Identification of chlamydial virulence factors awaits further research.

The aborting ewe is the primary source of infection for other susceptible sheep. Experimentally, pregnant ewes have been infected by all parenteral routes and by oral inoculation.^{278,332,350} Transmission of chlamydial infections probably occurs at the time of abortion when uterine fluids and placentas that contain large numbers of chlamydiae contaminate the environment and are ingested or inhaled by susceptible sheep.^{50,285,304,337,341} A recent study proposed that infection via tonsillar crypts may be the natural route of infection.¹⁸⁰ Chlamydiae may become established in the tonsil and spread by blood or lymph to other organs. Attempts to reproduce chlamydial abortion by infection of the female genital tract have been unsuccessful.^{85,388} *C. psittaci* can infect the genital tract of the ram, but venereal transmission is not considered an important factor in dissemination of this disease.^{20,39,219,285,337,340,341} Additionally, milk from infected ewes is not important in the transmission of chlamydiae to lambs.¹⁸¹ No biologic or mechanical vectors have been identified.³³⁷

Susceptible ewes usually are infected and abort during the same lambing season.^{37,50,278,390} However, it has been demonstrated that chlamydial infection contracted in the perinatal period may manifest itself in the next pregnancy and that ewe lambs could show

clinical disease in their first pregnancy from chlamydial infection transmitted to them before they become pregnant.^{238,388} Although the mechanism is unknown, chronic or latent chlamydial infections occur frequently.²⁰¹ Chlamydiae may survive in a latent form in the genital tract and lymphoid tissues.²⁵⁵ Other studies have shown that factors that slow down the chlamydial growth cycle, such as deprivation of metabolites needed for reproduction of chlamydia, incomplete inhibition of chlamydiae by antibiotic treatment, and lymphokines inhibit the maturation of reticulate bodies into elementary bodies and may favor persistent infections.^{155,157} The ability of chlamydiae to grow in macrophages and evade the host cellular immunity also may contribute to persistent infections.^{183,232,359} A cryptic form of chlamydiae that may revert to dividing RB when triggered by an unknown factor has been hypothesized to explain persistent infections.²⁵⁸

Pathogenesis

The ability to establish placental and fetal infection is a property of many chlamydial strains in several animal species, regardless of the type of placentation.^{323,337} Epithelial cells and infiltrating macrophages are the primary target cells.^{50,180,35, 170,238} Storz and coworkers studied the pathogenesis of chlamydial infection in ewes following intravenous experimental inoculation with an abortion strain of *C. psittaci*.^{22,342} Chlamydiae were cleared from the blood at a logarithmic rate as a function of time and size of inoculum.²² After initial clearance, chlamydiae could not be isolated from either the blood or somatic organs of ewes for approximately 18 hours.^{22,341} During the next 72 hours chlamydiae multiplied in many organs including the lung, liver, spleen, kidney and lymph nodes.^{22,183} A secondary chlamydemia

developed in all ewes 24 to 48 hours after initial inoculation and lasted for 2-3 days. The phase of secondary chlamydemia was more than twice as long in pregnant compared to nonpregnant ewes.²² Placental infection occurs during an episode of chlamydemia.^{22,285,338,341,342} Chlamydiae usually are cleared from maternal tissues while the infection progresses independently in the placenta and fetus.^{22,183,285,298,338,341,342} A brief chlamydemia has been identified in ewes following abortion and organisms may be shed intermittently in the feces.²⁷⁸

Severe placentitis is the predominant pathologic change in chlamydial abortion.^{1,204,212,268,332,337,341,349} Macroscopic changes in the placenta include hyperemia, hemorrhage, edema of the chorioallantois, necrosis of cotyledons, thickening of the intercotyledonary chorion, and accumulation of reddish brown to yellow flaky exudate in the uterine space (between the chorion and endometrium). These placental changes may be diffuse or limited to a few cotyledons. Stamp first described the microscopic changes in chlamydia infected ovine placentas, but Novilla and Jensen investigated placental lesions sequentially.^{50,268,332} Necrosis and suppurative inflammation with numerous chlamydial inclusions in chorionic trophoblasts are the predominant microscopic changes in chlamydia-infected placentas.

The fetus probably is infected following chlamydial invasion of the placental circulation.^{337,338,341} In one study, fetal infection was detected 3-5 days following intravenous inoculation of the ewe.³³⁶ Oral, conjunctival, or respiratory infections of the fetus are possible, but probably play minor roles. Amniotic and allantoic fluids may become infected

in late gestation subsequent to rupture of placental membranes.

The changes in ovine fetuses aborted due to infection with *C. psittaci* depend upon the stage of gestation and the duration of infection. Specific macroscopic lesions often are not observed.^{184,189,245,268,285,332,337,338,341} Advanced *in utero* autolytic changes are usually present in fetuses aborted in mid-gestation. Fetuses aborted near term are often fresh and in relatively good condition. Subcutaneous petechiae, mild hepato- and splenomegaly, hepatic congestion, whitish mottling of the liver, and enlarged edematous lymph nodes have occasionally been identified in chlamydia-infected ovine fetuses.^{190,337,338,341,349} Low birthweight and weakness are common findings in live chlamydia-infected lambs.

Accordingly, histologic lesions in the fetus are variable and often nonspecific.^{184,189,237,245,285,295} Pleocellular inflammatory infiltrates in portal areas of the fetal liver and widespread reticuloendothelial (lymphoid) hyperplasia are often the predominant microscopic changes. Multifocal necrosis with associated pyogranulomatous or non-suppurative inflammation in the liver, spleen, kidney, and lung and vasculitis and non-suppurative meningoencephalitis have been identified in naturally infected ovine fetuses.^{189,190,204,212,237,298,320,337,338,341,349}

Immune Responses and Vaccines

The nature and significance of immune mechanisms elicited by chlamydiae and their role in resistance are not fully understood. Ewes that abort due to infection with chlamydiae, usually conceive and do not abort again from this infection.³⁴¹ There is strong evidence of an enhanced immunological response and increased resistance to reinfection in sheep that

experienced chlamydial infection.^{22,85,342,387} Ewes previously exposed cleared chlamydiae from their blood at a significantly enhanced rate, and, in contrast to primary exposure, secondary chlamydia did not develop or was of much shorter duration.^{22,342} Enhanced clearance may reflect more efficient uptake and killing of chlamydiae by phagocytic cells and/or suppression of chlamydial multiplication in cells of somatic organs.

There are many chlamydial surface antigens that stimulate a humoral immune response. Immunoblotting studies with convalescent sheep abortion antiserum identified 30 polypeptides as antigenic both in abortion and intestinal isolates of *C. psittaci*.^{141,358} The 38-42 Kd MOMP and lipopolysaccharide are immunodominant antigens. The MOMP is prominent on the surface of all strains of chlamydiae and it contains species, subspecies, and serotype specific epitopes.⁶¹ Recent studies suggest that the MOMP may be an important immunoprotective determinant since it is capable of inducing neutralizing antibodies.^{141,160,270,359}

Chlamydial LPS is the genus-specific antigen that is present in all strains of this organism. The complement fixation test (CFT) that is widely used for serodiagnosis of chlamydial infections, detects antibodies to chlamydial LPS.^{337,341} Antibodies to chlamydial LPS are indicative of exposure, but do not protect against abortion.^{337,341} Chlamydial LPS possesses at least 3 distinct antigenic domains, 2 of which are shared by other gram-negative bacteria and 1 that is unique to the genus *Chlamydia*. The chemical nature of this chlamydia specific epitope has been described and recently synthesized.^(196,269,270)

Three cysteine-rich proteins of molecular weights 15 Kd, 60 Kd, 62 Kd may be important

in immunity to chlamydial infection in sheep.^{73,146,256} Additionally, two protein adhesins of 31 Kd and 18 Kd which appear to be eukaryotic cell binding proteins have been described.¹⁴⁶ These proteins are not denatured by heating and antibodies against the 18 Kd protein neutralizes chlamydial infectivity.¹⁸⁸ Recently, a highly immunodominant 26 Kd protein antigen was identified in intestinal strains of ovine *C. psittaci*, but has not been recognized in abortion isolates.¹⁴¹

Following infection in ewes, complement fixing antibody is produced against epitopes on chlamydial LPS. CFT titers peak at about 14-21 days after abortion and remain high for several weeks.^{189,245,337,341} Neutralizing antibody appears later, and titers remain higher for a longer time.^{232,250,341} Krauss and co-workers demonstrated that IgG1 is the dominant immunoglobulin subclass in ovine chlamydial infections.¹⁹⁷

Confusion surrounds the role of humoral immunity in defense against chlamydiae. Antibodies induced by chlamydial infection of sheep have both neutralizing and opsonizing functions.²⁹⁷ Several studies have suggested that humoral immunity, particularly neutralizing antibody, is necessary to protect against chlamydial abortion.^{3,14,130,141} However, Dawson and co-workers reported that high antibody titers were not an indication of protective immunity and in pregnant ewes there was an inverse relationship between delayed type hypersensitivity (DTH) and humoral responses.⁸⁵ Laboratory animal studies on passive transfer of antibodies also have produced conflicting results.^{52,53,379} A recent study demonstrated that immune sera and type-specific monoclonal antibodies could passively transfer resistance to placental and fetal colonization and to abortion and fetal loss in mice

intravenously challenged with abortion strains of *C. psittaci*.⁵¹

Infection of sheep with *C. psittaci* also stimulates cell mediated immunity.^{85,387,388} Cell mediated immune mechanisms may play a predominant role in resistance to infection with chlamydiae.^{51,130,237,379} Several studies have demonstrated that strong cell mediated immunity detected by DTH responses, prevents chlamydial abortions in ewes.^{85,387,388} DTH responses may enhance cytokine-activated phagocytosis of chlamydiae by macrophages. Byrne and colleagues demonstrated that infection with chlamydiae induced lymphokine mediated macrophage activation.²⁶¹ Migration inhibition of macrophages and lymphocyte proliferation also are enhanced following chlamydial infections.^{51,232} Alpha, beta, and gamma interferon have anti-chlamydiae activity, although gamma interferon is more potent.^{87,307} Oxygen-independent and dependent activation of macrophages against chlamydiae is enhanced by gamma-interferon (γ -INF).^{261,308} Infected cells activated by γ -INF inhibit replication of chlamydiae.^{54,55,111,261} The mechanism by which gamma interferon inhibits chlamydial growth appears to be different than that employed against viruses.²⁶¹ Catabolism of essential amino acids, particularly tryptophan, induced by γ -INF is one mechanism for this inhibition.⁵⁵ Protection against chlamydial abortion may require cooperation between the humoral and cell-mediated immune systems. Antibody dependent cell cytotoxicity and opsonisation are likely mechanisms.^{31,53}

Chlamydiae may modulate the host immune response by inducing cytokine release and/or by direct suppression of macrophage function.²³² Antigen from *C. psittaci* can directly inhibit in vitro lymphocyte proliferation.²⁰⁶ Immunosuppression associated with pregnancy is

a well recognized phenomenon and also may enhance chlamydial infection of the placenta.³⁶⁷

For more than thirty years, vaccines prepared from *C. psittaci* propagated in embryonated chicken eggs or in various cell cultures and inactivated with formalin, have been used in sheep to prevent chlamydial abortions. These inactivated vaccines were developed with little understanding of the underlying mechanisms of immunity and resistance to chlamydiae. Their efficacy has been variable and chlamydial abortions have occurred in vaccinated flocks.^{4,287,389} Investigations of both killed and live vaccines have continued in recent years. An alternative method to an effective vaccine has been pursued in France where a vaccine composed of a temperature sensitive mutant derived from an ovine abortion strain of *C. psittaci* was induced with nitroso-guanidine mutagenesis.³⁰¹ This vaccine induced good protection against challenge, but genetic instability was a problem. Recently, a vaccine composed of purified inactivated chlamydial EB has induced protection against challenge with a predominant antibody response against MOMP.¹⁴ A single dose of a MOMP-enriched subcellular preparation of detergent extracted EB protected against a subcutaneous challenge with a live homologous strain of chlamydiae at 70 days gestation.³⁵⁹ Additionally, the MOMP gene now has been sequenced and expressed in *E. coli* and a *Salmonella typhimurium* aro A mutant.¹⁶³

Antigens produced by these vectors are capable of eliciting both humoral and cellular immune responses and will hopefully lead to more efficient chlamydial vaccines in the future. Attention of investigators now is focused on the early events of chlamydia-host cell interaction functioning in attachment, entry, and mechanisms for avoiding chlamydial

destruction by lysosomes. Early events provide the best opportunities to abort chlamydial infection. Identification of surface antigens that initiate infection must be accomplished. Analysis of the surface proteins may help in identifying chlamydial antigens that result in the induction of humoral and cellular immunity.

Diagnostic Methods

Preferred specimens for diagnosis of chlamydial abortions are one or more fetuses and placentas that were recently collected and chilled until presentation at the laboratory. Useful laboratory techniques include histopathology, cytology, isolation of chlamydiae in cell culture or chicken embryos, serology, and various antigen detection procedures. The placenta is the diagnostic specimen of choice. Because the lesions are not uniformly distributed, the whole placenta should be examined for maximum diagnostic value.^{189,337} A tentative diagnosis of chlamydial abortion is often based upon the flock history, the presence of characteristic pathologic changes in placentas and fetuses, and demonstration of chlamydial EB in impression smears of placental tissues. The staining methods of Macchiavello or Gimenez are commonly used to demonstrate chlamydiae in placental smears and in histologic sections.^{83,189,192,337} Chlamydiae EB stain as bright red coccoid organisms and range from 0.3-0.5 microns in diameter.^{189,337}

A diagnosis of chlamydial abortion is confirmed by isolation and identification of the organism in chicken embryos or in tissue cell culture.^{178,189,192,245,337,339} However, these fastidious intracellular organisms are often difficult to isolate, especially under field conditions. They are readily inactivated in the course of intrauterine events that lead to fetal

death.^{214,337,339,345} Contamination by other bacteria and unfavorable transport conditions further reduce the viability of chlamydiae.³³¹ The inflamed placenta, which usually contains high numbers of viable organisms, is the tissue of choice for chlamydial isolation. Other fetal tissues often contain low numbers of viable chlamydiae.^{1,245}

The antibody responses of pregnant ewes experiencing chlamydial infections are characteristic and diagnostically indicative of recent infection.^{143,145,189,318,339} After inoculation of pregnant ewes an initial rise of complement-fixing antibodies is observed. The titers decline to low levels before abortion or parturition, provided these events occur later than 4 weeks after inoculation or exposure.³³⁹ Delivery of chlamydiae-infected fetuses stimulates a rapid rise in maternal antibody titer that reaches maximum levels two to three weeks postpartum.³⁴¹ Accordingly, paired serum samples, taken at the time of abortion and 2-3 weeks later, have a significant rise in titer if the abortion resulted from chlamydial infection.

The standard CFT detects antibodies to LPS the genus-specific antigen that is present in all strains of chlamydiae. It is relatively insensitive and does not distinguish antibodies produced to abortion strains of *C. psittaci* and strains associated with conjunctivitis, arthritis, and enteric infections.^{189,245,337,338} Enzyme-linked immunoabsorbent assays (ELISA) and indirect fluorescent antibody (IFA) tests have been developed as alternatives to the CFT, but have not been widely accepted.^{189,279,318} These serologic tests need to be refined so that specific antibody responses to abortion strains of *C. psittaci* can be identified. Fetuses aborted due to chlamydial infections often have elevated levels of immunoglobulins that are not reactive in the CFT, but have been identified with immunodiffusion, ELISA and

immunoperoxidase procedures.^{50,84,189,234,282,283,300,338}

Direct (non-culture) diagnostic tests including immunofluorescence techniques for cytologic specimens and formalin-fixed tissues,^{71,93,185,220,241,290,299,306,357,363} immunohistochemical procedures for detection of chlamydial inclusions in endometrial and cervical biopsies,²²⁴ ELISA methods,^{28,60,142,143,150,168,205,207,223,229,246,271,290,294,319,346,355,371,382} isotopic and non-isotopic nucleic acid probe and polymerase chain reaction assays,^{195,207,288} have been developed for detection of *C. trachomatis* infections in humans. These direct chlamydial tests have several advantages including speed, reduced cost, automation, and less stringent requirements for handling and transport of samples.

Until recently there have been few direct tests for detection of chlamydiae in specimens from animals. Soureau and Rodalakis developed an ELISA for detection of chlamydial antigen in vaginal swabs from aborting sheep and goats.³³¹ Later immunohistochemical and immunofluorescence procedures for demonstration of chlamydial antigen in formalin-fixed and frozen avian tissues and fecal specimens were introduced.^{127,256,277,394} Several ELISA tests for detection of *C. trachomatis* in human specimens have been evaluated for detection of chlamydial infections in cats,^{196,387} birds,^{136,292} and koalas.^{47,67-69,383} Generally, these tests have been less sensitive and less specific than chlamydial isolation in cell culture. Cross reactivity with other gram-negative bacteria has been a serious problem with some tests. An immunoassay designed specifically for detection of *C. psittaci* in ovine specimens and a differential polymerase chain reaction procedure for avian specimens are now available.^{169,291}

Other Infectious Causes of Ovine Abortion

Abortions, stillbirths, and weak lambs of low viability are important sources of economic loss to the sheep industry. These reproductive problems often have an infectious cause. Most placental infections occur via a hematogenous route. The type of placentation in sheep, with maternal septal tips intimately associated with placental hematomas, provides an accessible route to the placenta. Depending upon the stage of gestation, infection of susceptible ewes may result in abortions, stillbirths, and weak lambs. The common abortifacients will be discussed.

Organisms commonly associated with abortions in sheep include *Campylobacter fetus* var.fetus, *Campylobacter jejuni*, *Toxoplasma gondii*, and *Salmonella arizonae*. Less common infectious causes of reproductive failure in sheep include *Brucella ovis*, border disease virus, *Listeria* sp., and the rickettsia *Coxiella burnetti*. A wide variety of other bacteria, which are ubiquitous in the environment, have been associated with sporadic ovine abortions.

Campylobacteriosis

Ovine campylobacteriosis, also known as vibriosis, is caused by infection with *Campylobacter* which are small, curved, microaerophilic, gram-negative bacterial rods. Three species *Campylobacter jejuni*, *Campylobacter fetus* var.fetus, and occasionally *Campylobacter coli* are associated with reproductive disease in sheep.^{15,89,90,94,162,173,192} Abortion usually occurs in the last six weeks of gestation.^{89,192} The abortion rate may be as high as 70% in susceptible flocks. In flocks where the disease is endemic, 5-10% of the

ewes may be affected annually if no control methods are instituted.^{89,192}

C. fetus and *C. jejuni* may persist in the gallbladder and intestine of some sheep which intermittently shed these organisms in the feces. *C. jejuni* is commonly found in the intestinal tract of many species and can cause abortion or diarrhea in humans,^{194,226,291,326} dogs,^{127,291} cats,¹⁰⁴³ goats,⁹⁴ and cattle,^{125,291,292,370} as well as sheep.^{15,89,90,94,162,173,192} Several species of wild and domestic animals are potential reservoirs of *C. jejuni* and may be part of the transmission cycle of campylobacter infection in sheep.^{15,89,291} Susceptible sheep are often infected through contact with aborted fetuses, fetal membranes, and postpartal vaginal discharges.¹⁹² Dissemination of the organism through the flock is rapid and experimentally the incubation ranges from 8 to 60 days.^{89,162,173,192,218}

Aborted fetuses may be fresh or in various stages of decomposition. Multifocal and random areas of hepatic necrosis ranging from 0.5-2.0 cm in diameter, are highly suggestive of campylobacteriosis.^{89,162,184,192} Occasionally fibrinous pleuritis and peritonitis are present in the aborted fetus.¹⁹² Placental inflammation may not be apparent on gross examination, but microscopic lesions characterized by edema, hyperemia, neutrophilic inflammation, necrosis, and vasculitis with thrombosis occur consistently.^{89,162,173,192} Chorionic trophoblasts lining placentomal hematomas are the first placental sites involved. Colonies of bacteria distend the cytoplasm of chorionic trophoblasts, endothelial cells, and fill capillary lumens.^{89,162,173,192} Mild purulent bronchopneumonia and multifocal necrotizing hepatitis are characteristic microscopic changes.^{89,162,192}

A tentative diagnosis of campylobacter abortion is often based upon the flock history and

demonstration of characteristic lesions in the liver and lungs. The diagnosis is confirmed by isolation of this organism from the placenta, abomasal contents, and/or various fetal tissues.^{89,192}

Toxoplasmosis

Infections caused by *Toxoplasma gondii* are an important cause of reproductive failure in sheep. The incidence of toxoplasma abortion in a flock can range up to 40%.^{35,38,43,49,100,101,104,106-108,192,325} Depending upon the stage of pregnancy at the time of primary infection, *T. gondii* can cause embryonic and early fetal death, mummification, late-term abortions, stillbirths, weak lambs, or subclinical infection.^{38,103,104}

T. gondii is a common obligate intracellular protozoan with a two-stage asexual life cycle that can occur in most warm blooded animals, and a coccidian-type sexual cycle which is confined to the intestine of felids.^{48,49,103} The cat passes oocysts in its feces which sporulate on the ground to be picked up by other species. Sporulated oocysts can survive in the soil for over 500 days.¹⁰³ Sheep are infected when they eat feed containing infective oocysts; ingestion of as few as 200 oocysts may cause abortion in susceptible ewes.²³⁵

Ingested oocysts excyst in the digestive tract and released sporozoites penetrate the intestine, enter the blood and spread to the brain, liver, muscles, and the placenta. Fetal tissues are invaded during the second to third week after infection and abortion may occur 2-4 weeks later.³⁸

Multifocal areas of necrosis and mineralization in placental cotyledons is the characteristic lesion of ovine toxoplasmosis.^{35,49,102,103,108} The intercotyledonary placenta is not

affected.^{49,184,192} Usually there are no macroscopic lesions in fetuses aborted due to toxoplasmosis. Characteristic microscopic changes include multifocal necrosis and gliosis in the brain and scattered granulomas in visceral organs.^{49,103,184,192} *Toxoplasma* rarely are identified in histologic sections.

Diagnosis of toxoplasmosis usually is based upon identification of characteristic lesions in the placenta and brain. Demonstration of *Toxoplasma* antibodies in fetal fluids or precolostral lamb serum provides evidence of transplacental infection, however the absence of antibody is inconclusive.^{49,192} Because antibodies to *T. gondii* persist for several years, a high titer in an aborting ewe is not definite evidence of recent infection or that toxoplasmosis caused the abortion. Toxoplasmosis may be confirmed by mouse inoculation studies. Four to six weeks after mice are inoculated with placental or fetal tissues, examinations to demonstrate characteristic lesions, the organisms, or seroconversion to toxoplasmosis can be done.^{103,192}

Salmonellosis

Several species of *Salmonella* including *S. abortus-ovis*, *S. typhimurium*, *S. arizonae*, and *S. dublin* have been associated with abortion in ewes.^{90,213} *S. abortus-ovis*, a host specific serotype that is enzootic in certain areas of Europe, has not been identified in the U.S.⁽⁹⁰⁾

In the U.S. the host-adapted species *S. arizonae* is a relatively common cause of sporadic abortion in sheep. This organism may cause both intestinal and extra-intestinal infections with fecal, vaginal and nasal shedding.²⁹³ Ewes infected with *S. arizonae* usually are asymptomatic.^{215,293} The pathogenesis of *S. arizonae* abortions is poorly understood.

Experimentally, *S. arizonae* isolated from aborted ovine fetuses, did not induce disease in lambs or abortion in pregnant ewes.¹⁵³

Occasionally, other serotypes of salmonella, particularly *S. typhimurium*, have been associated with abortions in sheep. In a few instances, up to 60% of ewes infected with *S. typhimurium* have aborted.⁹⁰ Depression, fever and diarrhea may be observed in ewes infected with *S. typhimurium*. Overcrowding and other stresses may predispose to development of salmonella abortions. Placentitis and fetal septicemia results in fetal death. Ovine fetuses infected with salmonella usually are severely autolyzed and emphysematous. A diagnosis of salmonella abortion is based upon isolation of the organism from the fetus, placenta, and/or vaginal discharges.

Brucellosis

Brucella ovis is an important cause of epididymitis and infertility in rams and occasionally causes sporadic abortions, stillbirths, and weak lambs.^{192,210,210,211,250} *B. ovis* is transmitted venereally to the ewe. Ewes clear the infection after abortion and are not considered to be an important reservoir for spread of the organism to susceptible rams the following breeding season. *B. abortus*, important in cattle and other species including humans, only occasionally affects sheep and goats.^{17,184} *B. melitensis* infection is generally associated with goats, but sheep and other species are susceptible.²⁴⁷

It is recognized in the Mediterranean area, Africa, Central America and rarely in the U.S.⁽²⁴⁷⁾ Severe placentitis characterized by thickening, edema, and hyperemia of the intercotyledonary placenta and necrosis of cotyledons, is a consistent feature of brucellar

abortion.^{17,34,210,250} Typical histologic lesions include edema and diffuse suppurative inflammation of the chorioallantois with vasculitis and necrosis of chorionic villi.^{210,247,250}

Ovine fetuses aborted due to brucellar infection may be fresh or moderately autolyzed. Occasionally, fibrinous pleuritis or peritonitis are present. A purulent bronchopneumonia is a common microscopic change. Diagnosis of brucellar abortion is usually based upon the gross and microscopic placental and fetal lesions and by isolation of the organism.

Coxiella burnetti (Q Fever)

The rickettsia *Coxiella burnetti* is the cause of the zoonotic disease Q-fever.³³ It is an obligate intracellular organism, but in contrast to other members of the genus *Rickettsia*, it completes its life cycle in the endosomes of nucleated cells and is not dependent on arthropod transmission. This intracellular, gram-negative, bipolar rod-shaped bacteria is carried asymptotically by cattle, sheep, and goats. Additionally, many species of birds, wildlife, and domestic animals also can be infected.^{33,114} It is shed in milk, urine, feces and in higher numbers in the amniotic fluids and placenta.³³ Inhalation of the organism from contaminated environments is the most common mode of transmission.³³ Infection with *C. burnetti* has been associated with sporadic late term abortions in sheep and more commonly in goats.^{75,228,275,296,374}

Ovine fetuses aborted due to infection with infection with *C. burnetti* usually are severely autolyzed. Inflammation, necrosis, edema, and fibroplasia with mineralization of the intercotyledonary placenta are characteristic pathologic changes associated with infection with *C. burnetti*. Chorioallantoic trophoblasts contain intracytoplasmic colonies of

basophilic coccoid rickettsial organisms. Diagnosis is based upon gross and histologic placental lesions, serology, and isolation of the organisms in embryonated chicken eggs.³⁹⁶

Listeriosis

Infection with *Listeria monocytogenes* is responsible for sporadic and occasionally multiple abortions in sheep. The abortion rate from natural infection can range upward to 20%.³²⁸ The natural habitats of *L. monocytogenes* are the soil and the mammalian intestinal tract. *L. monocytogenes* has a predilection for the ruminant placenta, especially in later gestation.^{248,328} Because alkaline pH enhances the growth of *Listeria*, infections in ruminants are often associated with feeding poorly-preserved silage.³²⁸ Transmission by genital discharges may also occur.³²⁸

Genital listeric infection is characterized by placentitis, fetal death and abortion, stillbirths, and weak non-viable lambs.^{184,248} Macroscopic placental changes include edema, ulceration, necrosis of cotyledons, and abundant red-brown exudate on the chorionic surface. Aborted fetuses are usually severely autolyzed and catarrhal abomasitis, enteritis, widespread petechiae, and occasionally fibrinous pericarditis and/or peritonitis have been described. Fetal livers often contain multiple grayish-white foci ranging from 0.5 to 2 mm in diameter.^{184,328}

Suppurative inflammation with vasculitis and thrombosis are characteristic microscopic changes associated with listeric infection. Multifocal areas of coagulative necrosis with colonies of gram-positive coccobacillary bacteria and purulent to pyogranulomatous inflammation are present in the liver and occasionally in other organs. Hemorrhage and

neutrophilic infiltrates often are present in the lungs. *Listeria* are readily isolated from aborted fetuses.

Leptospirosis

Leptospiral abortions are uncommon in sheep. Sheep are relatively resistant to infection and are not considered to be primary reservoirs of leptospires.^{91,208} Leptospiral infection of susceptible pregnant ewes often results in an acute illness with icterus, anemia and/or a hemorrhagic syndrome. Abortions, stillbirths and weak lambs are associated with maternal illness. Only *L. pomona* has been isolated from sheep in the U.S., however, other serovars have been identified in other countries.^{193,208} Icterus, widespread subcutaneous petechiae, visceral congestion, and marked hepatomegaly are typical changes in fetuses aborted due to leptospirosis.²⁰⁸ Diagnosis of leptospirosis is based upon characteristic clinical signs, macroscopic fetal lesions, serology, immunofluorescence procedures and occasionally by isolation.¹⁹³

Other Bacterial Causes of Abortion

Bacterial species of widely varying pathogenicity have been associated with sporadic abortion in sheep. Included in this group are *Staphylococcus aureus*, *Streptococcus* sp., *Pasteurella multocida*, *Yersinia pseudotuberculosis*, *Bacillus* sp., *Bacteroides* sp., *Fusobacterium nucleatum*, *E. coli*, *Actinomyces pyogenes*, and *Hemophilus somnus*.^{91,186} Many of these organisms are ubiquitous in the environment. There are no distinctive characteristics of these abortions and neither placental nor fetal lesions are specific. In order to be considered the cause of an abortion, these organisms must be isolated in pure or almost

pure culture, inflammatory lesions must be present, and more likely causes must be eliminated.

Border Disease

Border disease has been reported in all major sheep raising countries and is an important cause of reproductive failure.^{8,25,262,362} It is caused by fetal infection with border disease virus (BDV), a pestivirus that is closely related to bovine viral diarrhea (BVD) and hog cholera viruses.^{117,266} Experimentally, BDV is also pathogenic for cattle, goats, and pigs.^{117,263} Cytopathic and non-cytopathic strains of BDV have been identified.

Susceptible ewes are infected by ingestion.^{26,263} The sheep fetus is susceptible to BDV from 18 to 130 days gestation and death occurs about 35 days after infection.^{24,92,263} Infection during the first trimester usually caused fetal death and abortion.^{24,70,266} In the second trimester, BDV may cause fetal death followed by mummification or abortion, stillbirths, malformations of the central nervous system (i.e., cerebellar hypoplasia and dysplasia, hydranencephaly, and porencephaly) abnormal fleece (i.e., "hairy shaker" or "fuzzy" lambs) and skin pigmentation, and the birth of small weak lambs of low viability.^{24,70,266} Abortion may occur at any stage of pregnancy and is most common during gestation days 90-120.⁹² Infections during the third trimester when the fetus is immunocompetent often are not significant.

Because of its variable manifestations diagnosis of border disease can be challenging. A reliable diagnosis is based upon the flock history, clinical signs, and specific lesions of the central nervous system (CNS) lesions.⁹² Histopathologic examination, serology,

immunofluorescent antibody tests of fetal tissues, and viral isolation from the kidney and spleen are useful laboratory procedures for diagnosis of border disease.

Other Viral Causes of Abortion

Infections with other viruses including Akabane, foot-and-mouth disease, rinderpest, sheep pox, and ovine progressive pneumonia can cause fetal death and abortion in sheep.^{79,184} Infection of susceptible ewes with bluetongue virus during the first half of gestation may result in resorption, abortion, mummification, stillborn and weak lambs and CNS malformations including hydranencephaly, porencephaly, and hydrocephalus.²⁷⁷ Recently, infections with Cache Valley virus, a bunyavirus of the Bunyamwera serogroup, has caused epizootics of arthrogryposis, CNS malformations, early embryonic and fetal death, mummification, stillbirth, and weak lambs.^{77,110}

Neosporosis (Bovine Protozoal Abortion)

Neosporosis or Bovine Protozoal Abortion is a newly recognized fetal infection caused by an intracellular coccidian parasite that is similar to *Neospora caninum*.²⁹ Recent reports indicate that neosporosis is a common cause of abortion in dairy cattle and occasionally goats.²⁹ Experimentally, infection of pregnant ewes with this organism resulted in abortion of autolyzed fetuses 25 and 26 days post-inoculation.¹⁰⁹ Multifocal necrosis and gliosis in the brain, myocarditis, myositis, and necrotizing placentitis are characteristic microscopic changes. A diagnosis of neosporosis is confirmed with immunohistochemical methods and/or ultrastructural evaluation.²⁹

Non-Infectious Causes of Abortion

A number of non-infectious causes of sporadic abortions/reproductive failure also affect sheep. Genetic and/or developmental abnormalities, deficiency of vitamin E or selenium, rumen acidosis, pregnancy toxemia, nitrate toxicosis, iodine deficiency, inadequate nutrition, and severe maternal stress or illness are examples, but will not be discussed further.

3. GROSS AND MICROSCOPIC PATHOLOGY OF OVINE FETUSES INFECTED WITH *CHLAMYDIA PSITTACI*

Introduction

Chlamydial abortion in ewes, also known as enzootic abortion of ewes (EAE), was first described in Scotland in 1936, but it was 1950 before Stamp identified *Chlamydia psittaci* as the etiologic agent.^{140,332} In many areas it is the most commonly diagnosed infectious cause of reproductive failure in sheep.^{3,4,68,161,198,219,237,245,286,320,321,337}

Chlamydial infection of susceptible pregnant ewes may cause abortion, stillbirths, or weak lambs.^{4,219,237,268,323,337,341} Late term abortions without well-defined clinical signs is the most common presentation, but behavioral changes and abundant yellow-brown vaginal discharge may be present shortly before the abortion.^{1,329} On initial exposure the incidence of abortion may be as high as 30% of the flock.^{1,2,4} Once infection is established, abortions in 1-5% of ewes annually are common.^{323,341} Fertility and subsequent pregnancies usually are not affected.^{323,337}

The aborting ewe is the primary source of infection for other susceptible sheep. Most ewes are infected and abort during the same lambing season.^{37,50,278,390} Transmission of chlamydial infections probably occurs at the time of abortion when uterine fluids and placentas that contain large numbers of chlamydiae contaminate the environment and are ingested or inhaled by susceptible sheep.^{50,285,304,337,341} A recent study suggested that infection via tonsillar crypts may be the natural route of infection.¹⁸⁰ Chlamydiae may become established in the tonsil and spread by blood or lymph to other organs.¹⁸⁰ Placental infection

occurs during an episode of chlamydemia.^{22,285,338,341,342} Chlamydiae usually are cleared from maternal tissues while the infection progresses independently in the placenta and fetus.^{22,183,285,298,338,341,342,342}

The purpose of this study was to characterize the gross and microscopic lesions associated with chlamydial infections in ovine placentas and fetuses.

Materials and Methods

Sheep

Forty-six 8-14 month old crossbred non-pregnant(open) ewes were employed. All ewes lacked serologic titers to chlamydiae with the complement fixation test (CFT)(National Veterinary Services Laboratories, Ames, Iowa) and there was no evidence of chlamydiosis in the flock history. Estrus was synchronized with progesterone acetate impregnated vaginal pessaries(sponges) (Chrono-gest[®], Intervet Int. B.V., Boxmeer, Holland). Pessaries were removed after 10 days and each ewe received 7 ml of pregnant mare serum gonadotrophin (Theriogenology Section, Veterinary Teaching Hospital, College of Veterinary Medicine, Iowa State University) by intramuscular injection. Ewes were in estrus 24-48 hours later and were bred naturally with two 12-18 month-old sexually mature Suffolk rams. Pregnancy was confirmed with real-time ultrasound and gestational age was determined by the date of last service.

Inoculum

Second passage of strain OSP of *C. psittaci*, an ovine abortion isolate maintained at the National Animal Disease Center, was used in this study. Chlamydiae were propagated in Vero cells (American Type Culture Collection, Rockville, MD) in the presence of cyclohexamide (Sigma Chemical Co., St. Louis, MO), harvested, suspended in sucrose phosphate glutamate buffer,³³¹ and frozen in aliquots. Aliquots of chlamydiae were thawed and diluted 1:10 in 0.1 M phosphate buffered saline (PBS)(pH 7.2). Titration studies determined that this inoculum contained approximately 10^6 chlamydiae per ml.

Inoculation Procedures

Forty pregnant ewes received 3 ml of inoculum intravenously in the jugular vein during gestation days 85 to 110. Six pregnant ewes received an intravenous injection of 3 ml of PBS (pH 7.2) and served as controls. Rectal temperatures were recorded before inoculation and daily thereafter for 5-7 days. Ewes were maintained in isolation until necropsy.

Necropsy and Tissue Collection

Ewes inoculated with *C. psittaci* were necropsied at selected times from 4 to 60 days after inoculation (Table 1). Control ewes were necropsied 14, 21, 28, 35, 42 and 45 days after inoculation.

At necropsy, gravid uteri were exposed by ventral laparotomies on anesthetized, heparinized ewes. After sodium pentobarbital (Ft. Dodge Laboratories, Ft. Dodge, IA) overdose uterine veins were severed and erythrocytes were flushed with Hanks' balanced salt solution (pH 7.4). After removal from vaginal attachments, the gravid horns were opened by

a longitudinal incision along the lesser curvature, and the fetus was exteriorized. The amnion was incised after a 5 ml sample of amniotic fluid was withdrawn for chlamydial isolation procedures. Fetuses were separated from their placental attachment by severing the umbilical cord. The intact placenta was examined for gross lesions and samples for microbiologic evaluation were collected before fixative perfusion. When no macroscopic changes were identified, eight placentomes chosen from the ends and one from the middle part of the placenta were collected for microbiologic evaluation. Uteri were fixed by intraarterial perfusion with 2-4 liters of 1.25% glutaraldehyde and 1.0% para-formaldehyde in 0.1 M cacodylate buffer (pH 7.4) into the middle uterine arteries over a period of 5 to 10 minutes. Uterine and placental tissues were collected for light and electron microscopy. Uteri of ewes which had aborted were not perfused due to the reduced size of uterine arteries.

Tissue Preparation

Representative sections of uterus, placentome, spleen, liver, cervix, vagina, vulva and lymph nodes (inguinal, pelvic, mammary) from each ewe and sections of fetal lung, adrenal, liver, kidney, spleen, heart, thymus, brain, lymph nodes and interplacentomal placenta were collected and fixed in 10% neutral buffered formalin. Tissues were dehydrated in graded series of ethanol, cleared with xylene, infiltrated and embedded in paraffin, cut at 5-6 μm , and stained with hematoxylin and eosin (H&E) for microscopic examination. Selected tissues were stained with modified Gimenez procedure.¹³²

Bacteriology

Stomach contents, lung, liver, and placenta were cultured aerobically and anaerobically on 5% bovine blood agar and campylobacter agar (Bacto Campylobacter Agar Kit Skirrow, Difco Laboratories, Detroit MI)(Iowa Veterinary Diagnostic Laboratory and Clinical Microbiology, Iowa State University). Bacterial isolates were identified by standard methods.⁶⁷ Stomach contents were examined for leptospire and campylobacter-like organisms with darkfield microscopy. When placental inflammation was identified macroscopically, smears of placental cotyledons were made on standard glass microscope slides, air dried, and were stained by the Gimenez method.¹³²

Chlamydial isolation: Samples of fetal lung, placenta, liver, spleen, lymph nodes (mesenteric, hepatic), kidney, abomasal contents, amniotic fluids, meconium, and conjunctival swabs along with maternal liver, lung, lymph nodes (mesenteric, inguinal, pelvic), spleen and feces were collected for chlamydial isolation procedures.

Approximately 1 g of each tissue was minced with a mortar and pestle and a 10% (weight/volume) suspension was prepared in cell culture medium composed of Eagle's minimum essential medium with Earle's balanced salts containing 20 mmol HEPES(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid); 5% fetal bovine serum; 5.4 mg/liter of glucose; 292 mg/liter of glutamine; 2 µl of amphotericin B; 500 µg/ml each of streptomycin, vancomycin, and kanamycin; and 0.5 µg of cyclohexamide. Samples were refrigerated overnight at 4°C. The fluid was centrifuged for 10 minutes at 700 x g at 20°C, the middle 1 ml of the supernatant was removed and 200 µl was inoculated into each of 2 wells of

a 96-well plate containing 24-h-old confluent Vero cell monolayers. Four plates were inoculated to provide cultures for examination at different times and/or for repassage. Inoculated plates were centrifuged at 1,000 x g for 60 minutes at 35°C. The inoculum was removed, fresh medium was added, and plates were incubated at 37°C in 4% CO₂.

One plate was fixed with a 50% acetone-50% methanol mixture for 5-10 minutes on days 3 and 6. Monolayers were stained and examined for chlamydial inclusions with an indirect fluorescent antibody (FA) technique using a group-reactive mouse monoclonal anti-chlamydiae antibody and a fluorescein-conjugated anti-mouse IgG immunoglobulin (IgG-heavy and light chain specific)(Organon Teknika, Malvern, PA) at a dilution of 1:30.

Serology

Serum was collected from each ewe before inoculation and biweekly until necropsy. Fetal heart blood and/or thoracic fluids were collected at necropsy and centrifuged. The supernatant fluid was stored at -20°C until tested. Maternal sera was examined for chlamydial antibodies with a CFT (National Veterinary Services Laboratories, Ames, Iowa).

Fetal serum and thoracic fluids were evaluated for levels of IgG immunoglobulins with a single radial immunodiffusion method.^{118,161,225} Briefly, 7.5 ml of 1% agarose in PBS (pH 7.2) containing 0.1 ml of goat anti-sheep IgG (heavy and light chain-3.7 mg/ml) (Chemical Credential, ICN Immunobiologicals, Lisle, IL) was melted and stabilized at 50°C. Melted agar was poured into immunodiffusion plates (Chemical Credential, ICN Immunobiologicals, Lisle, IL) and allowed to harden for 60 minutes. After hardening, 3 mm wells were cut with a gel cutter (Chemical Credential, ICN Immunobiologicals, Lisle,

IL). Dilutions of sheep IgG (200, 100, 20, 2 mg/dl)(Chemical Credential, ICN Immunobiologicals, Lisle, IL) in PBS (pH 7.2) served as controls. Five microliters of control and test sera/fluids were added to indicated wells and covered plates were allowed to set undisturbed at room temperature (25° C) for 18-20 hours. Precipitin rings were directly measured. A standard curve (ring diameters [x-axis] versus their known concentration on semi-log graph paper) was constructed using the diluted control values. The IgG concentration of test samples was determined by reading against the standard curve.

Fetal fluids with concentrations of IgG greater than 20 mg/dl were tested for antibodies to *Toxoplasma gondii* with a latex agglutination test (Toxotest-MT, Eichen Chemical Co. LTD., TANABE USA INC., San Diego, CA.) and to chlamydiae with CFT (National Veterinary Services Laboratories, Ames, IA).

Results

Clinical Observations

All ewes developed a febrile response (rectal temperatures >40° C) 1-2 days post-inoculation (DPI) and feed consumption was slightly reduced. Body temperatures remained elevated for 3-7 days. Control ewes were afebrile and there was no evidence of clinical disease.

Eight ewes aborted 4-11 DPI (Table 1). Fetuses were passed with no maternal straining or preparturient signs. They were autolyzed and probably had died 1-2 days before presentation.

Three ewes died 5 days after infection (Table 1). There was no clinical indication of a

life threatening systemic disease. Each ewe had received aspirin (500 grains [32.5 grams]/day) orally for 2 days prior to their deaths.

Three ewes aborted at 43, 58, and 59 DPI (Table 1). A yellow-brown vaginal discharge was noted shortly before the abortion. One ewe lambbed at 60 DPI (Table 1) and its single lamb was small (approximately 2.3 kg) and weak.

Gross Pathology

Placentas: Placental lesions were identified in 20 of 40 inoculated ewes (33 of 64 placentas) (Table 1). The distinction between mild, moderate and severe placentitis was based on the extent of placental involvement. When inflammatory changes involved less than 20% of the placenta, they were considered mild. The placentitis was considered severe when more than 50% of the placenta was affected. Otherwise, the placentitis was classified as moderate.

Mild placentitis was characterized by slight edema, hyperemia, and small localized accumulations of white to yellow-brown exudate in placentomes and periplacentomes. Extensive edema, hemorrhage and abundant exudate on the chorionic surfaces were characteristic features of severe inflammation. Exudate on the chorion varied from a thick, brown, bloody fluid to a dry yellow-white layer up to 0.5 cm in thickness (Figs. 1,2). Beneath the exudate, particularly in the interplacentomes, the chorioallantoic membrane was necrotic. Placentomal lesions varied from multiple yellow-brown foci of necrosis and inflammation to complete necrosis (Fig 2). Necrotic cotyledons detached easily from caruncles in advanced stages of the disease. Gimenez stained impression smears of inflamed

cotyledons revealed numerous small ($< 1 \mu\text{m}$ in diameter), red, coccoid organisms that were compatible with chlamydial elementary bodies.

There were no macroscopic changes in placentas from 14 ewes examined 4 to 17 DPI. Mild placentitis was present in placentas from 6 of 9 ewes examined 18-30 DPI (Table 1). Moderate to severe placentitis was identified in 14 of 17 ewes examined 32-60 DPI (Table 1). No abnormalities were identified in placentas and uteri from control ewes.

Fetuses: Sixty-four fetuses from ewes inoculated with *C. psittaci* were examined and specific macroscopic changes were not identified. Lymph nodes from most late-term fetuses examined 40-60 DPI were enlarged and often had distinct cortices. Subcutaneous and visceral petechiae were noted in aborted fetuses. Extensive subcutaneous edema and hemorrhage, abundant serosanguineous fluid in body cavities, and pink-red discoloration of tissues were typical changes in fetuses aborted 4-11 days after inoculation. These changes were compatible with *in utero* autolysis and suggested that fetal death had occurred 1-2 days before the abortion.

Ewes: Accumulations of thick yellow-brown exudate on caruncles and the endometrium was associated with severe placental inflammation. Small amounts of exudate mixed with blood were present in vaginas of aborting ewes. Pelvic and inguinal lymph nodes of ewes with advanced placental inflammation were slightly enlarged. Uteri from ewes with mild placental changes were unremarkable. Lesions were not observed in respiratory, cardiac, gastrointestinal, mammary, or urinary systems of inoculated or control ewes.

Necropsy examination of the 3 dead ewes revealed generalized visceral congestion,

severe diffuse pulmonary edema, and the tracheas and bronchi were filled with blood-tinged foam (also consistent with severe pulmonary edema). Moderate to advanced autolytic changes were noted in all tissues.

Histopathology

Placentomes: Chlamydial infection was identified microscopically in 35 placentas from 21 ewes (Table 2). Placentas from inoculated ewes examined 4-11 days post inoculation (DPI) and from control ewes were unremarkable. Microscopic changes were first identified in placentomes of a ewe examined 14 DPI (Figs. 3,4). In this placenta, erythrophagocytic chorionic trophoblasts lining placentomal hematomas were packed with basophilic organisms that were less than 0.5 μm in diameter and stained dark red with a modified Gimenez stain. The size, morphology, staining characteristics, and intracytoplasmic location of these organisms were compatible with chlamydiae. Minimal inflammation was associated with these organisms (Figs. 3,4).

The extent and severity of chlamydial infection and inflammation increased with duration of infection. In placentomes from a ewe examined 18 DPI, many chorionic trophoblasts lining placentomal hematomas contained chlamydial inclusions. Multifocally, chlamydia-infected trophoblasts were necrotic and/or sloughing into the hematomas. Necrosis and ulceration of the chorion was associated with infiltrates of inflammatory cells, predominantly neutrophils with variable numbers of macrophages, in the subepithelial mesenchymal tissues (hilar stroma)(Fig. 5). In placentomes examined ≥ 28 DPI, there was extensive necrosis and ulceration of chorionic trophoblasts lining placentomal hematomas (Figs. 6,7).

Hematomas contained abundant exudate that consisted of desquamated degenerate chlamydiae-filled chorionic trophoblasts, cellular debris, free chlamydiae, neutrophils and macrophages (Figs. 6-10). Severe inflammation resulted in lysis of erythrocytes in hematomas. Subepithelial mesenchymal tissues (hilar stroma) were edematous and infiltrated with a mixed population of inflammatory cells, predominantly macrophages and neutrophils (Figs. 8-10). Additionally, chorionic villi were edematous, segmentally denuded and/or necrotic, and heavily infiltrated with neutrophils and macrophages (Fig 11). Maternal septa were necrotic and often obscured by exudate. Segmental vasculitis characterized by neutrophilic infiltrates of the media and adventitia and thrombosis was present multifocally in the hilar and villous stroma. Placentomal infection with chlamydiae was well established before involvement of the peri- and interplacentomes was identified.

Periplacentome and interplacentome: Inflammation of the periplacentome was detected at 21 DPI and the interplacentome at 24 DPI (Figs. 12). There was a similar progression of events in that infection of chorioallantoic trophoblasts with chlamydiae was followed by degeneration and ulceration, intense infiltrates of neutrophils, and accumulation of exudate on the ulcerated chorioallantois and in uterine spaces (lumens). Extensive ulceration and intense inflammation of the intercotyledonary chorion was a consistent finding in chlamydiae-infected placentas examined 32-60 DPI. Intercotyledonary mesenchymal tissues (stroma) were markedly thickened due to edema, deposition of fibrin, and heavy infiltrates of neutrophils (Figs. 13,14). Vasculitis and thrombosis were prominent features of severely inflamed placentas (Fig. 15). Only chorionic trophoblasts contained

intracellular chlamydiae. However, numerous free chlamydiae were present in exudate in hematomas and uterine spaces.

Ewes: Necrosis of caruncles, ulceration of the endometrium, accumulations of purulent exudate in endometrial glands, and intense infiltrates of a mixed population of inflammatory cells in the lamina propria/submucosa (strata compactum and spongiosum) often accompanied severe placental inflammation (Figs. 16,17,18). Chlamydial inclusions were present in endometrial mucosal epithelium where the inflamed chorioallantois was in apposition to maternal tissues. Variable amounts of inflammatory exudate was present on the luminal surface. Neither inflammation nor chlamydiae were identified in the cervixes or vaginas. Pelvic and inguinal lymph nodes were hyperplastic. No abnormalities were identified in other tissues from inoculated ewes. Tissues from control ewes were unremarkable.

Fetuses: Microscopic changes were identified in tissues from 24 of 38 fetuses infected with chlamydiae (Table 2). A summary of these changes is given in Table 3. No abnormalities were identified in tissues from fetuses examined 4-17 DPI. A few small necrotic foci with minimal inflammation were detected in the livers of two fetuses examined 18 DPI. Microscopic changes were more common in chlamydiae-infected ovine fetuses examined ≥ 32 DPI.

Lymphoid hyperplasia was common change in chlamydiae-infected fetuses examined more than 30 DPI. Lymph nodes from these fetuses, unlike controls, had distinctly demarcated cortices with numerous lymphoid follicles that contained large, lightly stained

germinal centers (Fig. 19). Medullary cords were well developed and paracortices also were expanded. Neutrophils were numerous in the medullary cords of these nodes and the medullary sinuses contained many cells, mostly macrophages, neutrophils, and lymphocytes. In contrast, lymphoid tissues of control fetuses contained few lymphoid follicles, few cells were present in medullary sinuses, and neutrophils were not observed.

Infiltrates of mononuclear inflammatory cells, predominantly macrophages with fewer lymphocytes, in portal areas of the liver was another common finding in chlamydiae-infected fetuses examined 30-60 DPI (Fig 20). These portal infiltrates were more extensive in later stages of infection and often extended into the adjacent acinar parenchyma. In some livers there were multifocal discrete collections of large macrophages with abundant eosinophilic cytoplasm (granulomas)(Fig. 22). Multifocal and random areas of necrosis were first noted in two fetuses examined at 18 DPI, but also were more common in older fetuses (Fig. 21). Characteristically, these necrotic foci contained erythrocytes, cell debris, and variable infiltrates of neutrophils, and macrophages. Other microscopic changes in fetal tissues included scattered interstitial infiltrates of neutrophils and mononuclear cells in the myocardium (Fig. 23), multifocal necrosis and inflammation in the spleen (Fig. 24), perivascular infiltrates of mononuclear cells in the choroid plexus (Fig. 25), scattered foci of necrosis and gliosis in the brain (Fig. 26), and mild interstitial pneumonia characterized by thickening of alveolar septae due to infiltrates of mononuclear cells (Table 3). Chlamydiae were not identified in fetal tissues.

Serology

IgG immunoglobulins: Sera from fetuses infected with chlamydiae contained increased concentrations of IgG (mean=99 mg/dl). Base-line IgG immunoglobulin levels in clinically normal unstimulated ovine fetuses range from 10 mg/dl at 77 days of gestation to 22 mg/dl at birth.³¹⁰ IgG levels in serum from fetuses examined < 24 days were not increased. Increased levels of IgG were detected in fluids from 6 fetuses infected with chlamydiae and examined 24-30 DPI (mean = 43 mg/dl; range 20-70 mg/dl). High levels of IgG were detected in serum from 22 fetuses examined 31-60 DPI (mean IgG =139 mg/dl, range 55-300 mg/dl). IgG levels in 10 fetuses from normal control ewes and 26 non-infected fetuses from experimentally inoculated ewes were less than 20 mg/dl (mean = 12 mg/dl).

Complement fixation-chlamydiae: All ewes inoculated with *C. psittaci* developed CFT titers of 1:40 to 1:320 by 21 days post-infection. Control ewes remained seronegative. Fetal sera/thoracic fluids with IgG levels \geq 30 mg/dl were evaluated for chlamydiae specific antibodies with the CFT and results were negative.

Toxoplasmosis: Sera from 28 fetuses with IgG levels \geq 30 mg/dl were examined for antibodies to *T. gondii* and results were negative.

Bacteriology

C. psittaci was the only infectious agent identified. Chlamydiae were isolated from 38 of 64 placentas (Tables 4,5). High numbers chlamydiae were isolated from all placentas with macroscopic lesions. Low numbers of chlamydiae were isolated from placentomes

examined at 4,6, and 14 DPI.

Low numbers of chlamydiae were isolated from one or more tissues from 18 fetuses (Tables 4,5). No chlamydiae were isolated from tissues from fetuses examined less than 17 DPI. Generally, chlamydial isolation was more successful from tissues of fetuses examined 40-60 DPI (Table 5). Conjunctival swabs, lung, spleen, amniotic fluids, and liver proved to be the best specimens for chlamydial isolation.

Chlamydiae were isolated in low numbers from tissues from 18 of 40 inoculated ewes (Table 6). No chlamydiae were isolated from ewes examined after 17 DPI. The spleen and lymph nodes were the best specimens for chlamydial isolation.

Virology: Direct fluorescent antibody tests and virus isolation procedures for border disease virus were negative.

Table 1. Clinical signs and necropsy observations of ewes inoculated with *Chlamydia psittaci* by time of necropsy

Number Ewes	Necropsy PID ^a	Clinical Signs	Degree of Placentitis ^b	Number fetuses
1	4	Aborted	None	1
1	5	Aborted	None	2
3	5	Died	None	4
1	6	None	None	1
2	7	Aborted	None	4
1	9	Aborted	None	2
2	10	Aborted	None	2
1	11	Aborted	None	1
1	14	None	None	2
1	17	None	None	1
1	18	None	Mild	2
1	19	None	None	1
1	20	None	Mild	2
1	21	None	Mild	2
1	22	None	None	2
1	24	None	Mild	1
1	27	None	Mild	3
1	28	None	Mild/Moderate	2
1	30	None	None	2
1	32	None	Moderate	1
1	34	None	Moderate	1
1	38	None	None	2
1	39	None	Moderate	2
1	40	None	Severe	1
1	41	None	Moderate	2
1	43	Aborted	Severe	1
1	46	None	None	2
1	47	None	Moderate	2
1	49	None	Severe	2
1	52	None	None	1
1	56	None	Moderate	2
2	58	Aborted(1)	Severe	3
1	59	Aborted	Severe	2
2	60	Lambd	Severe	3

^a Post-inoculation days.placentas

^b Criteria for degree of placentitis defined in gross lesion results.

Table 2. Incidence of microscopic lesions in ovine fetuses and placentas infected with *Chlamydia psittaci* arranged by time of necropsy

Days ^a PI	Number ^b Fetuses	Microscopic Lesions ^c	
		Placentas	Fetal Tissues
4-10	2	0	0
11-20	6	6	2
21-30	8	8	9
31-40	5	5	16
41-50	7	7	14
51-60	10	9	21
Total	38	35	62

^a Days post-inoculation with *C. psittaci*.

^b Ovine fetuses infected with *C. psittaci*-confirmed by isolation in cell culture and/or demonstration of characteristic gross and microscopic lesions.

^c Total number of tissues with microscopic lesions.

Table 3. Microscopic lesions in ovine fetuses infected with *Chlamydia psittaci*

Lymphoid hyperplasia	17/38 ^a
Portal infiltrates-liver	15/38
Necrosis-liver	12/38
Pneumonia-interstitial	6/38
Necrosis-spleen	4/36
Myocarditis	3/38
Encephalitis	2/38

^a Data expressed as number of fetuses with lesions/numbers of fetuses infected with chlamydiae .

Table 4. Chlamydiae isolation results from ovine fetal tissues from ewes inoculated with *Chlamydia psittaci*

Tissue	Isolation
Placentas	38/64 ^a
Conjunctiva ^b	14/64
Spleen	14/64
Amniotic fluids	12/64
Lung	12/64
Liver	11/64
Lymph nodes	10/64
Abomasal contents	9/64
Kidney	9/64
Meconium	1/64

^a Data are expressed as number of ovine fetuses from which *C. psittaci* was isolated/number of fetuses evaluated.

^b Conjunctival swabs

Table 5. Chlamydial isolation results from ovine fetuses infected with *Chlamydia psittaci* arranged by time of necropsy

Days PI ^a	Number Fetuses	Isolation of <i>C. psittaci</i>				
		Placentome	Spleen	Lung	Liver	Kidney
4-10	16	2 ^b	0 ^b	0 ^b	0 ^b	0 ^b
11-20	9	6	1	0	1	0
21-30	11	8	3	1	2	1
31-40	7	5	4	2	2	1
41-50	9	7	2	2	2	1
51-60	11	9	4	7	4	6
Total	63	37	14	12	11	9

^a Post-inoculation

^b Figures expressed as numbers of isolations per number of fetuses evaluated.

Amniotic Fluids	Conjunctiva	Abomasal Fluids	Meconium	Lymph Nodes
0 ^b	0 ^b	0 ^b	0 ^b	0 ^b
0	0	0	0	0
3	0	1	0	0
2	3	1	0	3
3	4	3	1	3
4	7	4	0	4
12	14	9	1	10

Table 6. Chlamydiae isolation results from ewes inoculated with *Chlamydia psittaci*

Tissue	Isolation ^a
Lymph nodes ^b	16/40
Spleen	8/40
Lung	6/40
Kidney	3/40
Feces	2/40
Liver	0/40

^a Data expressed as number of ewes from which chlamydiae was isolated/numbers of ewes inoculated with chlamydiae.

^b Lymph nodes included mesenteric, internal iliac, supramammary, mandibular and retropharyngeal.

Fig. 1--Placenta of a ewe inoculated intravenously with 10^6 *C. psittaci*

and examined on day 43 post-inoculation. Note the severe placentitis characterized by diffuse hyperemia, edema, and abundant yellow-brown exudate on the chorioallantoic membrane.

Fig. 2--Placenta of a ewe inoculated intravenously with 10^6 *C. psittaci*

and examined on day 58 post-inoculation. Note the severe placentitis with necrosis of cotyledons, thickening of intercotyledonary placenta, and abundant red-brown exudate on chorioallantoic membrane.

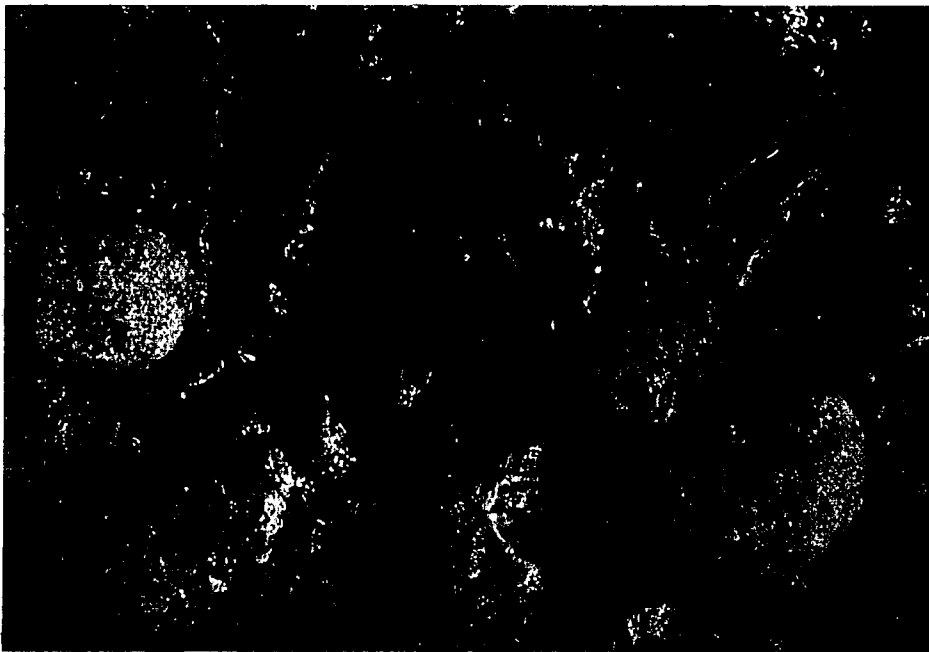
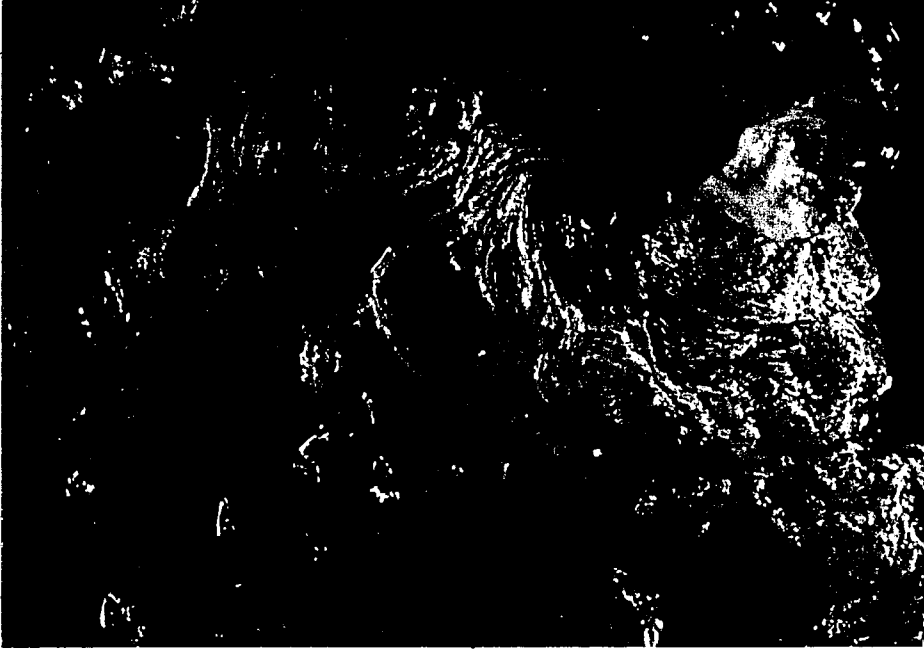


Fig. 3--Placentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on day 14 post-inoculation. Note that erythrophagocytic chorionic trophoblasts lining placentomal hematoma (HE) are packed with chlamydiae (chlamydial inclusions)(arrows) and minimal inflammation of subepithelial mesenchymal tissues (stroma) (SM). Magnification = 100 x.

Fig. 4-- Placentome from Fig. 3. Note that erythrophagocytic chorionic trophoblasts lining placentomal hematoma (HE) are packed with chlamydiae (chlamydial inclusions)(arrows) and there are no inflammatory infiltrates in subepithelial mesenchymal tissues(stroma) (SM). Magnification = 400 x.

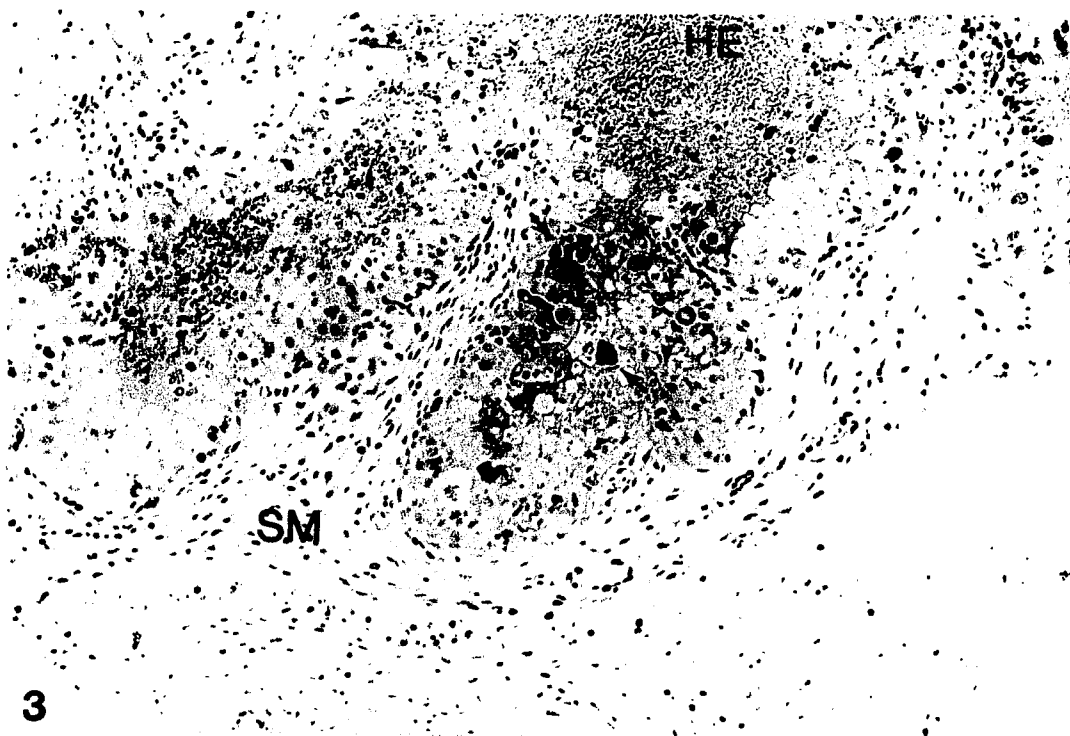


Fig. 5--Placentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on day 18 post-inoculation. Note that erythrophagocytic chorionic trophoblasts of placentomal hematoma (HE) contain numerous chlamydial inclusions (arrows), there is focal necrosis and ulceration of chorionic trophoblasts, edema, and infiltrates of inflammatory cells in the subepithelial mesenchymal tissues (stroma)(SM). Magnification = 100 x.

Fig. 6--Hematoma from a placentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on day 28 post-inoculation. Note that the chorion is ulcerated, hematoma (HE) contains neutrophils, cell debris, and numerous desquamated degenerate chlamydiae-infected trophoblasts, and mesenchymal tissues (stroma) (SM) are edematous and contain infiltrates of inflammatory cells. Magnification = 100 x.

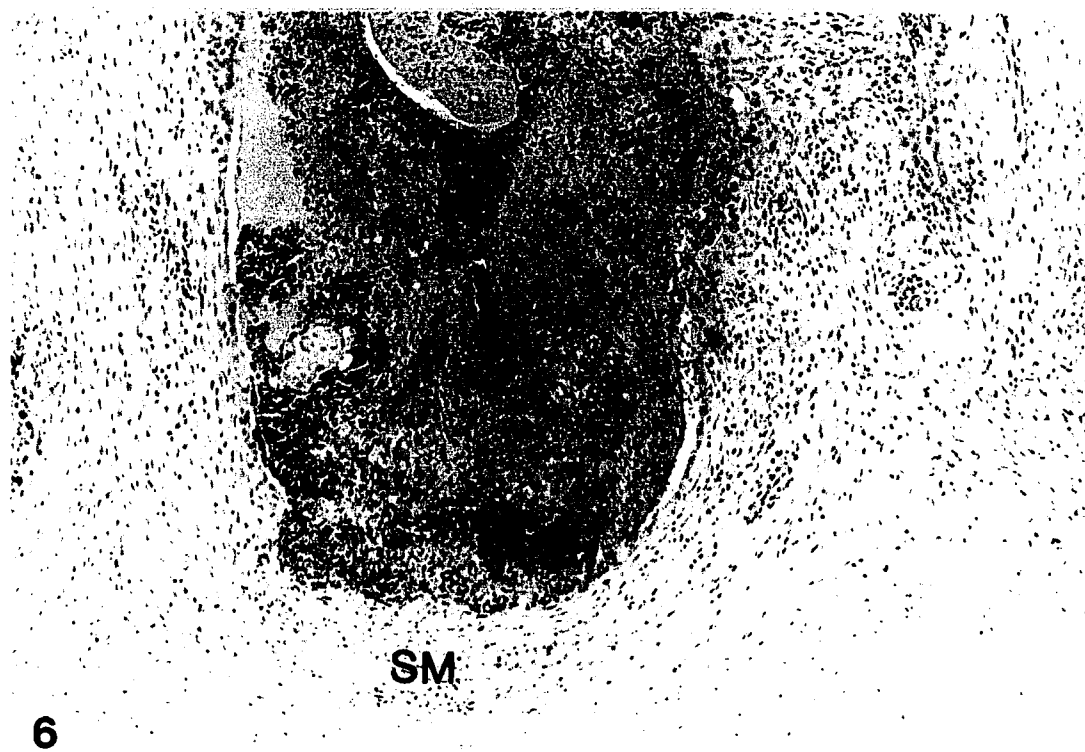
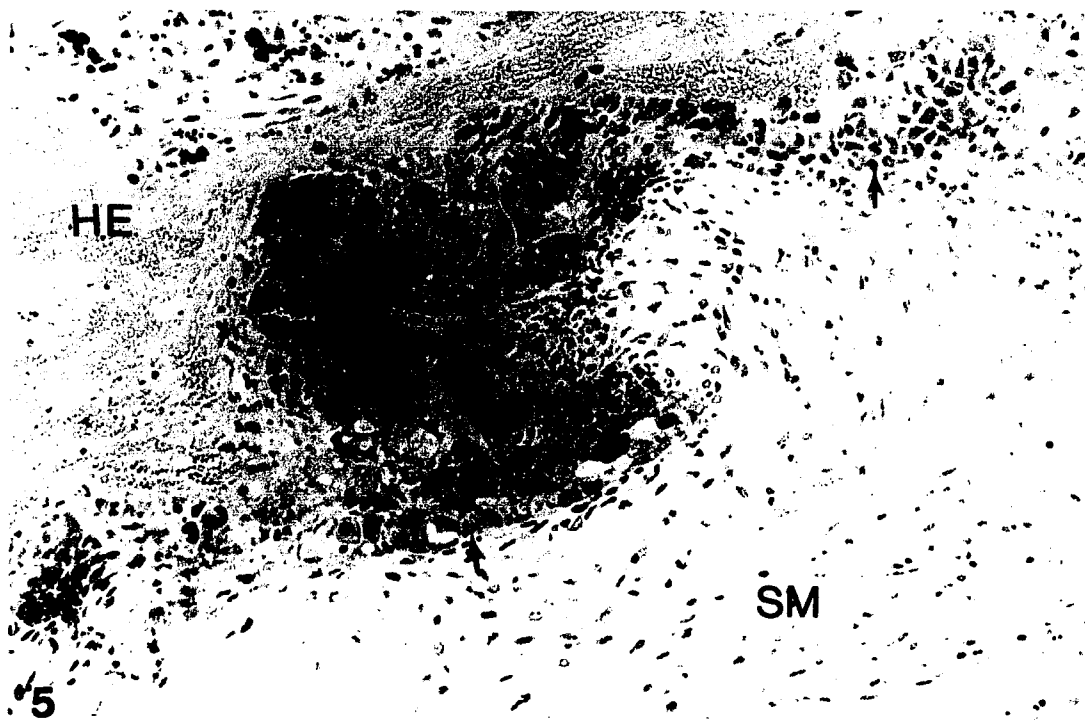
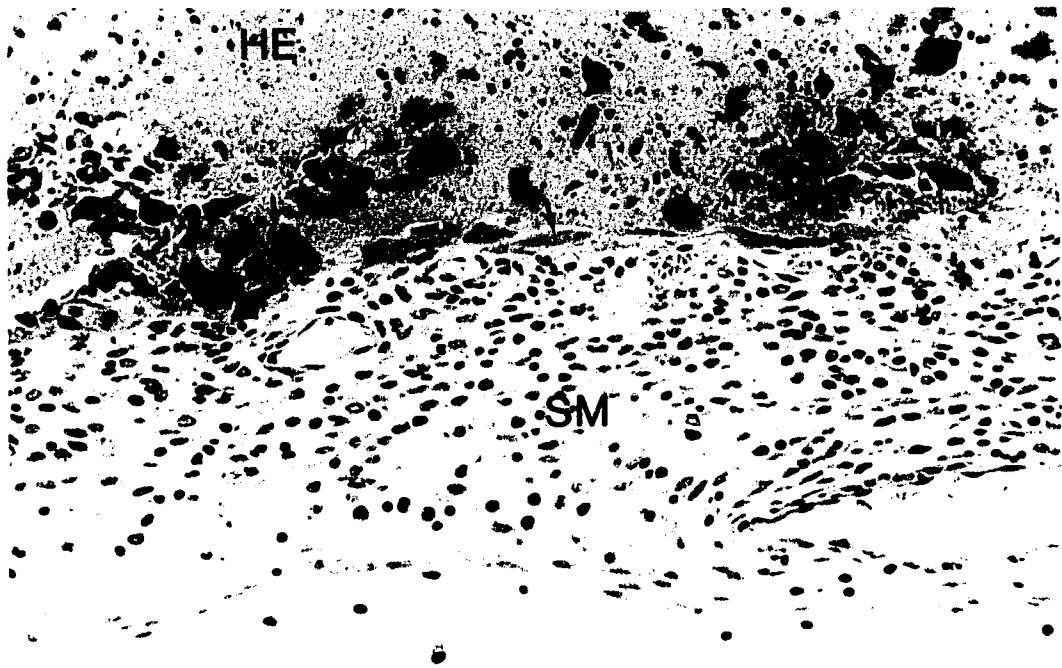
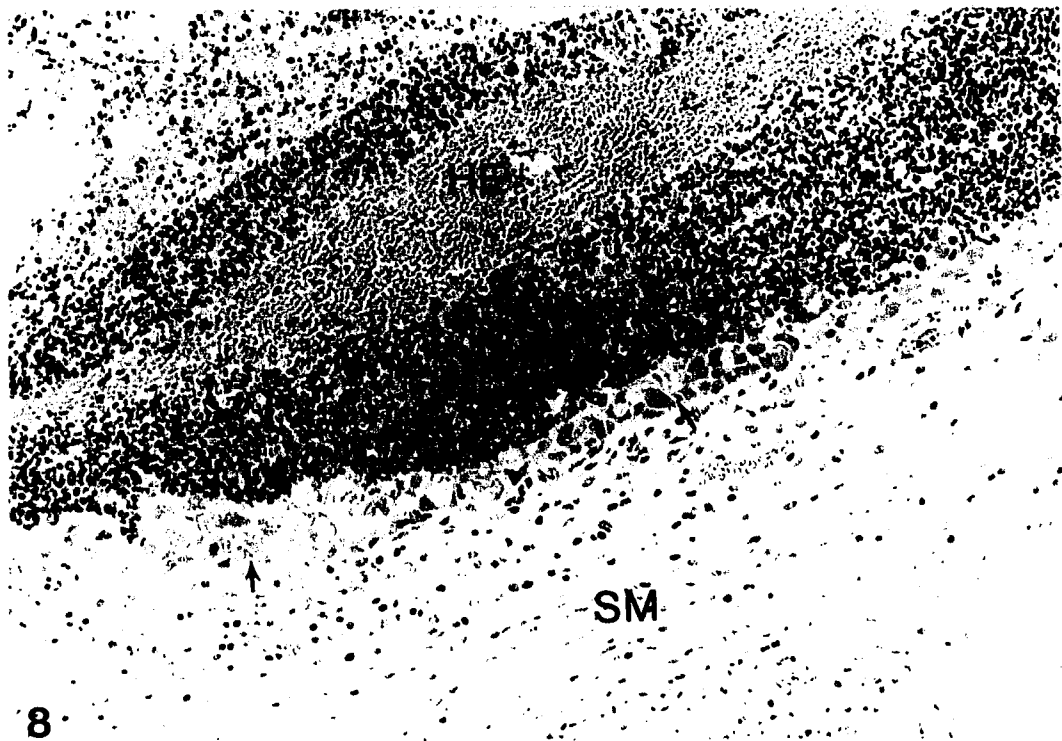


Fig. 7--Placentomal hematoma from Fig. 6. The chorion is ulcerated and hematoma(HE) contains numerous desquamated degenerate chorionic trophoblasts that are filled chlamydiae(arrows). The subepithelial mesenchymal tissues (stroma)(SM) are edematous and contain infiltrates of mixed population of inflammatory cells. Magnification = 400 x.

Fig 8--Placentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on day 38 post-inoculation. Note that many chorionic trophoblasts contain chlamydial inclusions (arrows), hematoma (HE) contains abundant exudate containing numerous neutrophils, and infiltrates of inflammatory cells in subepithelial mesenchymal tissues (stroma)(SM). Magnification = 160 x.



7



8

Fig 9--Placentome from Fig 8. Note that chorion of placentomal hematoma is diffusely ulcerated, subepithelial mesenchymal tissues (stroma) (SM) contain intense infiltrates of inflammatory cells, and abundant exudate on chorionic surface and in hematomas (HE). Magnification = 200 x.

Fig 10--Placentome from Fig 8. Note that there is extensive ulceration of chorionic epithelium lining placentomal hematomas, subepithelial mesenchymal tissues (stroma)(SM) are heavily infiltrated with a mixed population of inflammatory cells, and numerous desquamated degenerate trophoblasts containing chlamydial inclusions in hematomas (HE). Magnification = 200 x.

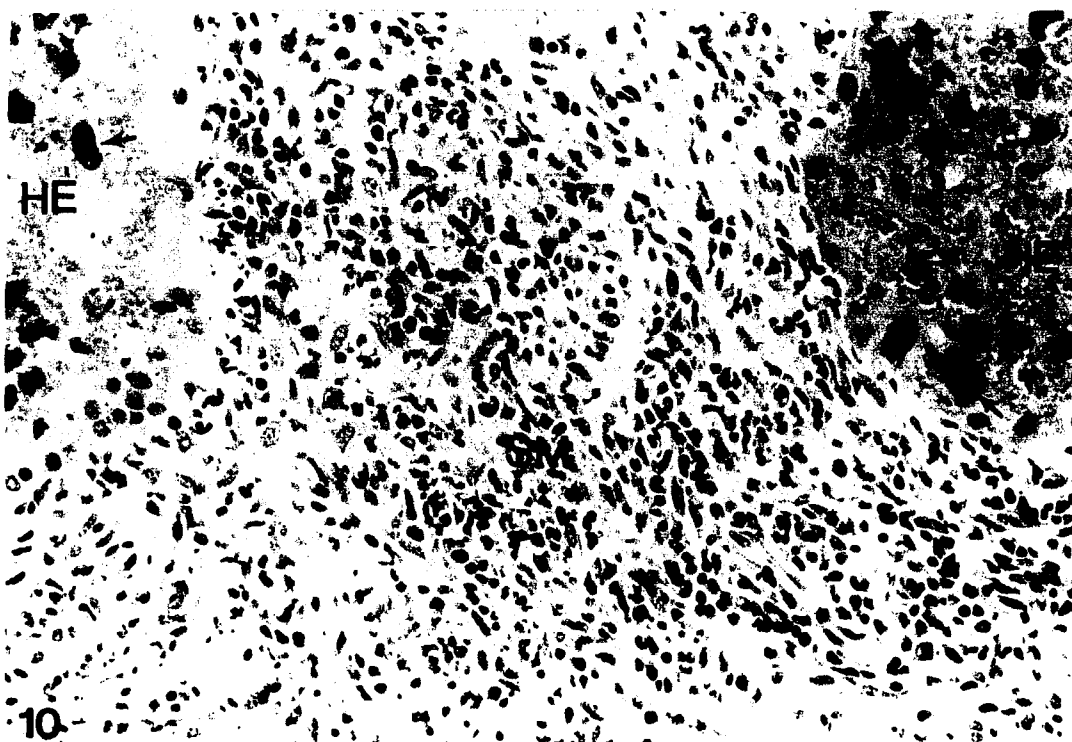
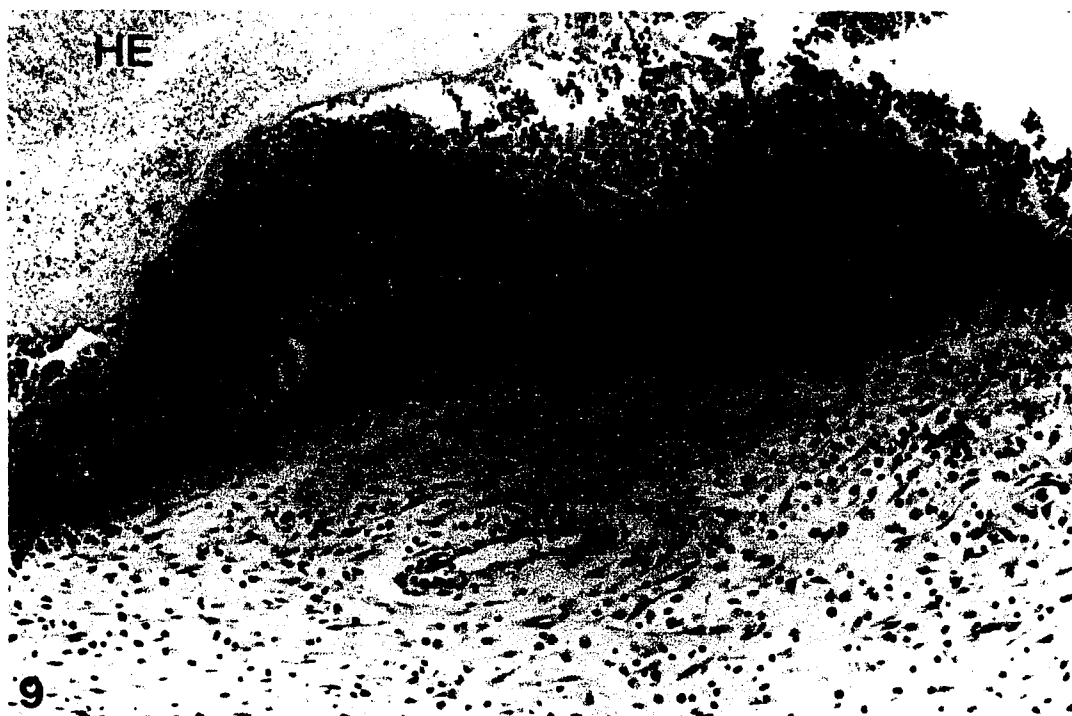


Fig. 11--Chorionic villus (CV) from placentome in Fig. 8. Note the diffuse ulceration of chorionic trophoblasts (arrows), intense infiltrates of inflammatory cells in subepithelial mesenchymal tissues (stroma), edema, and abundant inflammatory exudate in intervillous (IV) spaces. Magnification = 160 x.

Fig. 12--Periplacentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on day 24 post-inoculation. Note the extensive ulceration of chorioallantoic trophoblasts (arrows), intense infiltrates of inflammatory cells in subepithelial mesenchymal tissues (stroma)(SM), and abundant exudate composed of leukocytes and numerous desquamated degenerate chlamydiae-infected trophoblasts in uterine space (lumen) (US).

Magnification = 160 x.

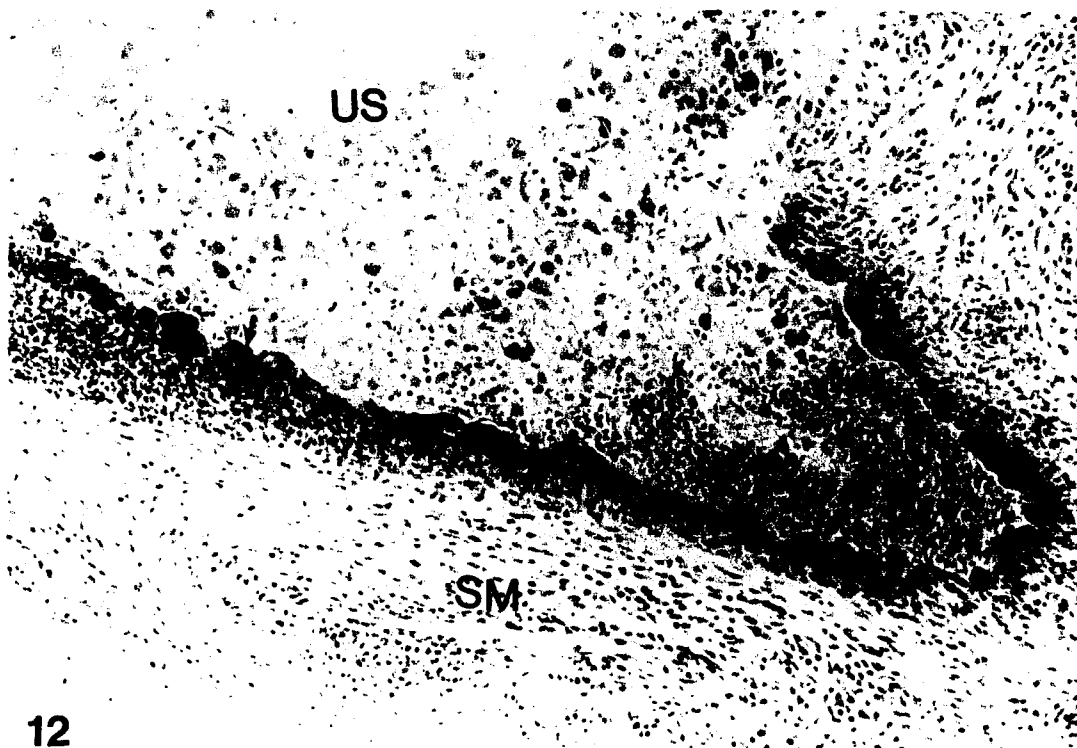
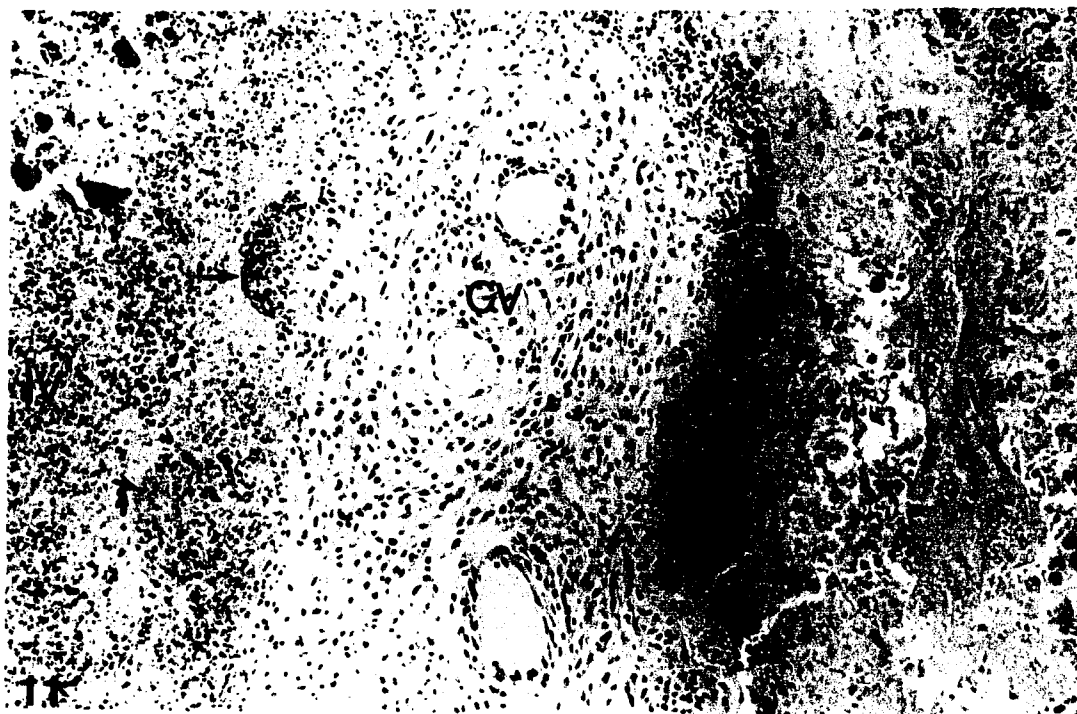
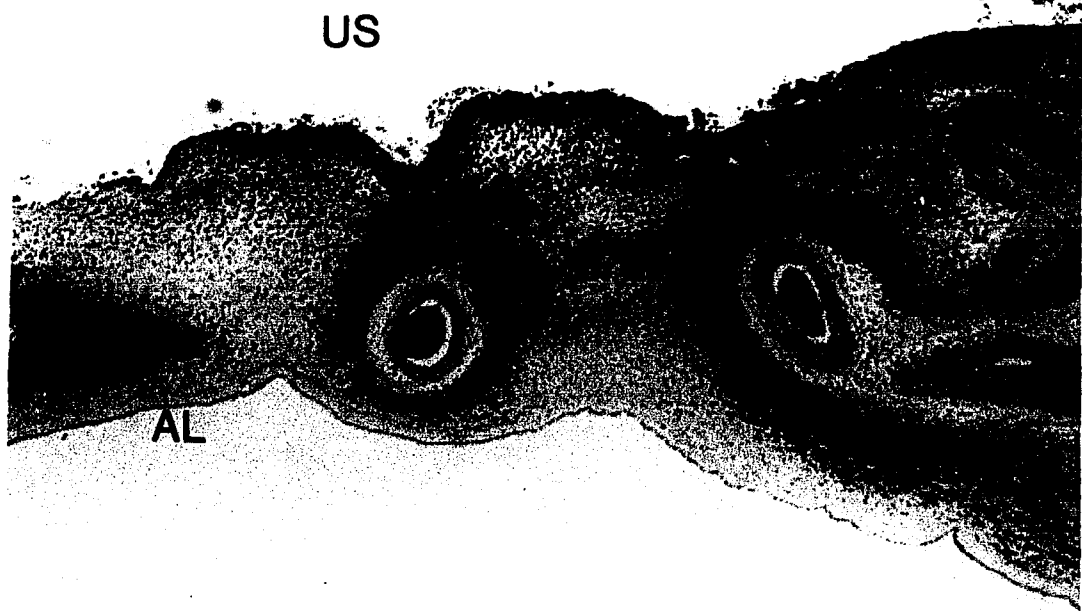
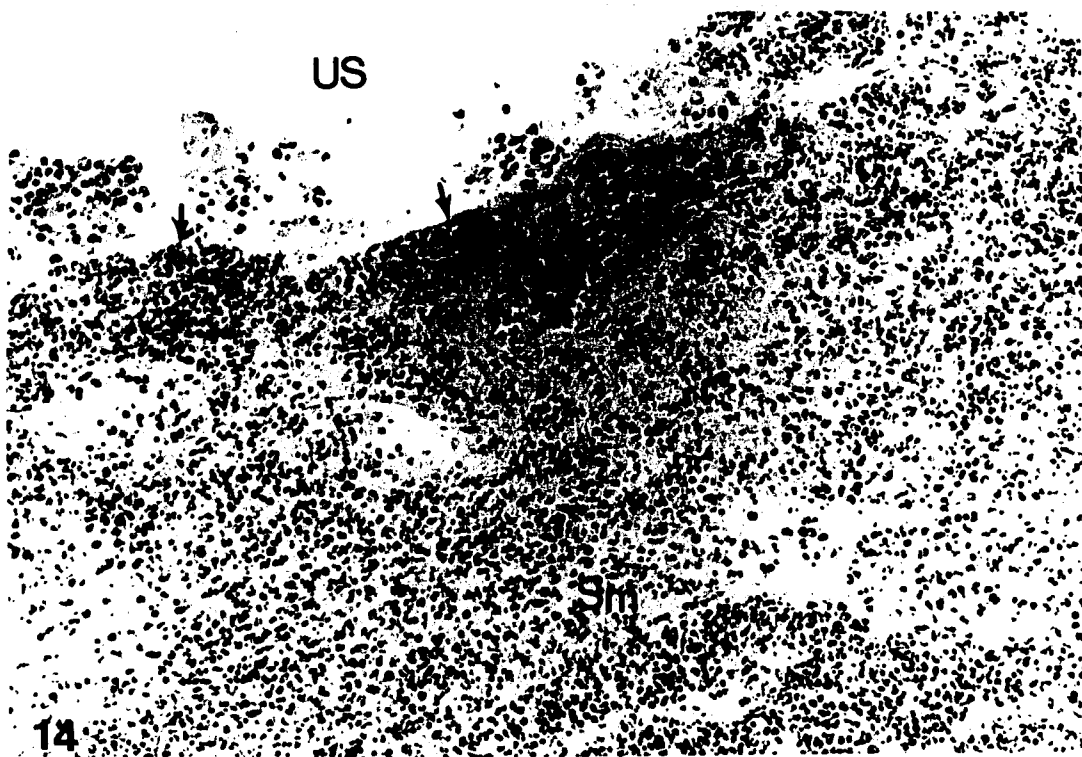


Fig. 13--Intercotyledonary placenta of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on post-inoculation day 56. Note that the chorion (CH) is diffusely ulcerated and that mesenchymal tissues (stroma) (SM) contain intense infiltrates of inflammatory cells. Allantois (AL). Magnification = 25 x.

Fig. 14--Intercotyledonary placenta from Fig. 13. The chorion is ulcerated (arrows) and there is necrosis, diffuse edema, and intense infiltrates of inflammatory cells in mesenchymal tissues (stroma) (Sm). Uterine space (lumen) (US) contains abundant exudate composed of necrotic cell debris and leukocytes. Magnification = 200 x.



13



14

Fig. 15--Intercotyledonary placenta from Fig. 13. Note the necrosis and intense infiltrates of inflammatory cells, predominantly neutrophils, in mesenchymal tissues (stroma)(SM) and vascular thrombosis (arrows). Magnification = 200 x.

Fig. 16--Pericaruncular endometrium (EN) of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on post-inoculation day 43. Uterine space (US) contains abundant inflammatory exudate composed of cell debris and numerous leukocytes. The endometrium is characterized by heavy infiltrates of a mixed population of inflammatory cells in the lamina propria/submucosa (stratum compactum/spongiosum) and exocytosis of neutrophils in the epithelium. Magnification = 160 x.

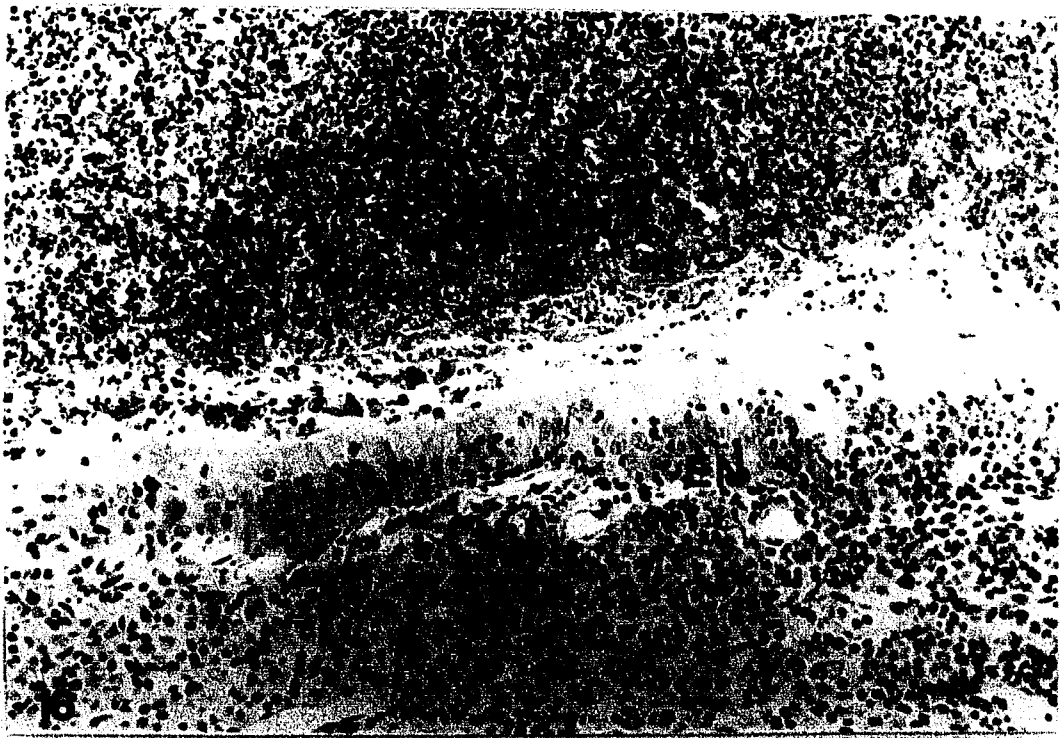
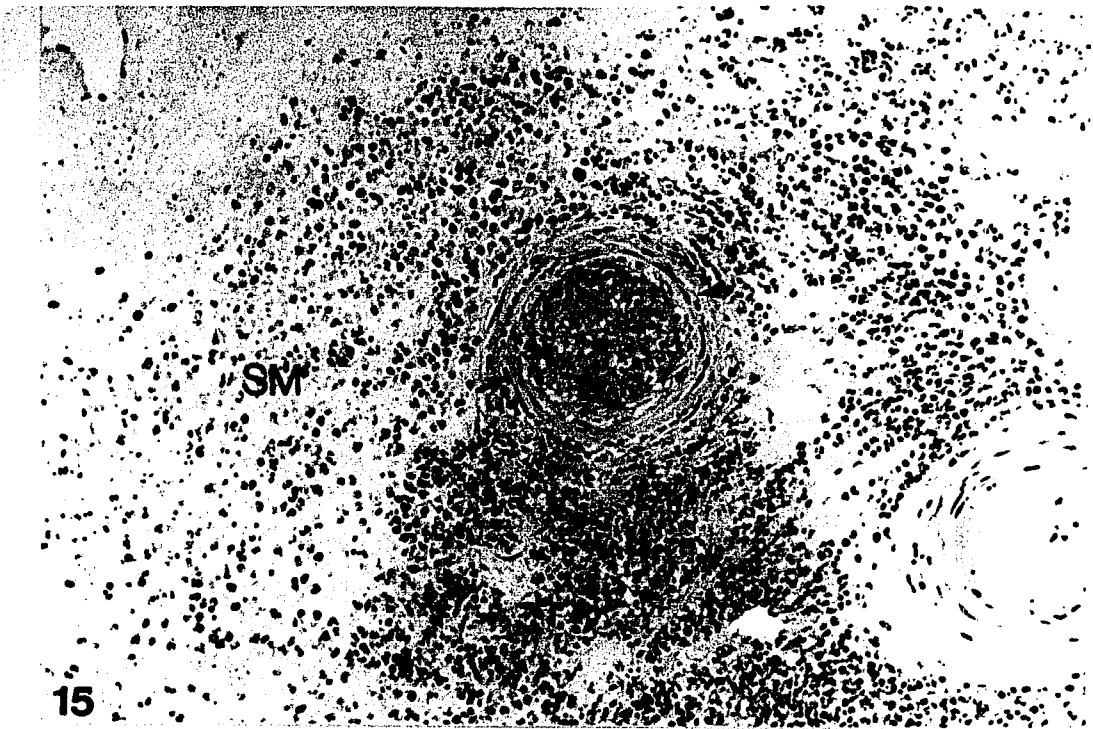


Fig 17-- Endometrium of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined 47 days post-inoculation. Note that the endometrium is ulcerated (arrows), infiltrates of a mixed population of inflammatory cells in the lamina propria/submucosa (stratum compactum/spongiosum), and exocytosis of neutrophils in uterine glands. Magnification = 200 x.

Fig 18--Uterine endometrium of a ewe inoculated intravenously with 10^6 *C.psittaci* and examined 56 days post-inoculation. Note the extensive infiltrates of mononuclear cells, predominantly lymphocytes, around uterine glands. Magnification = 200 x.

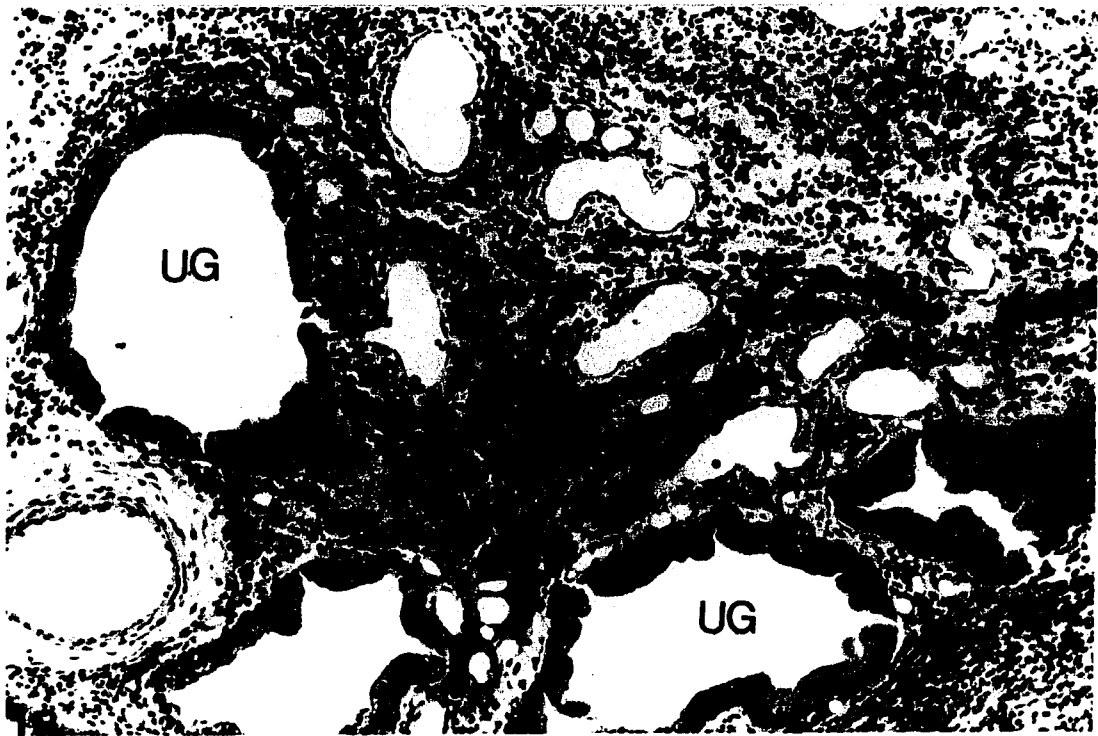


Fig. 19--Lymph node of an ovine fetus from a ewe inoculated intravenously with 10^6 *C.psittaci* and examined 43 days post-inoculation. Note the large lymphoid follicles with prominent germinal centers in the cortex. Magnification = 200 x.

Fig. 20--Liver of an ovine fetus from a ewe inoculated intravenously with 10^6 *C.psittaci* and examined 41 days post inoculation. Note the infiltrates of mononuclear cells, predominantly macrophages and lymphocytes, in portal areas. Magnification = 160 x.

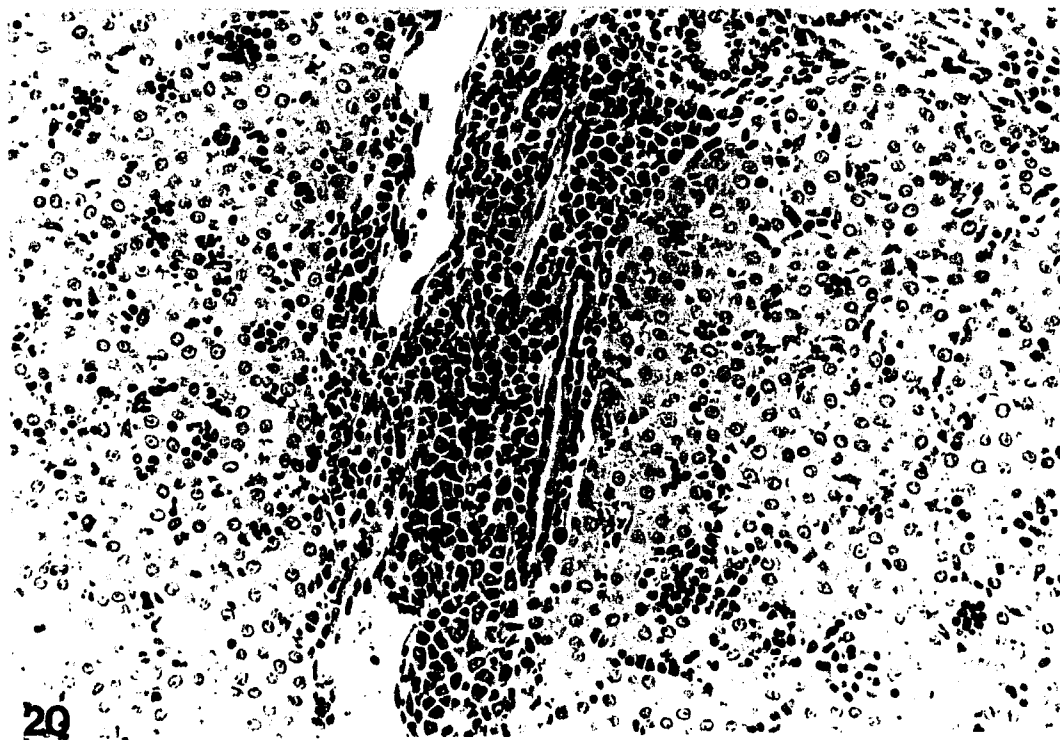
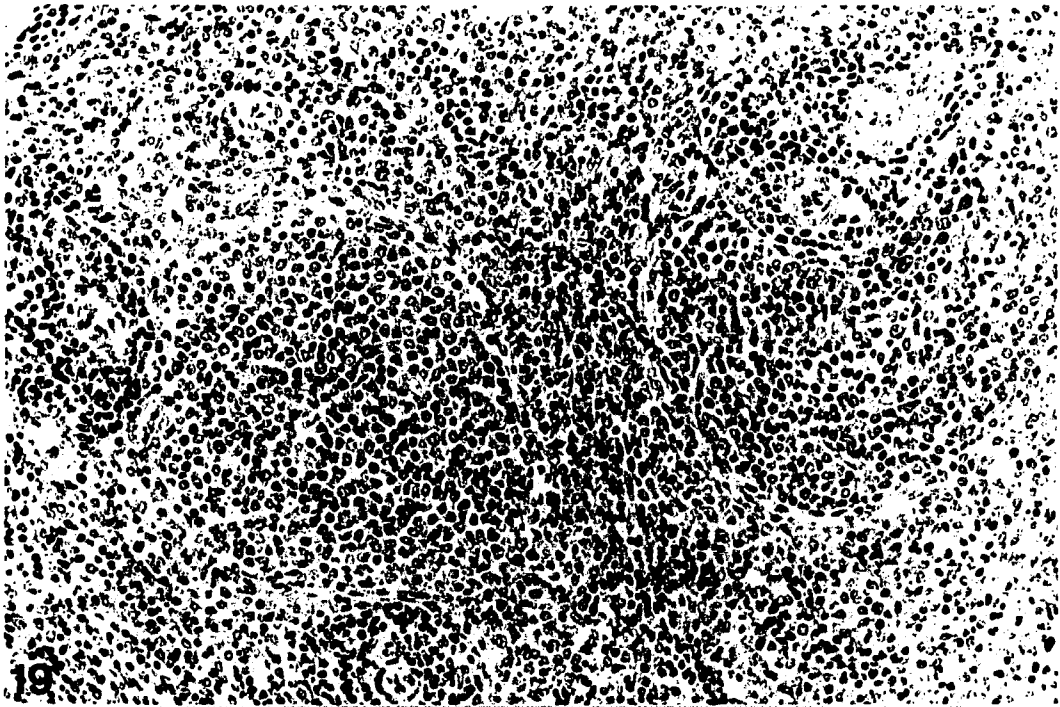


Fig. 21--Liver from Fig. 20. Note the focal area of necrosis (arrows) and mild infiltrates of neutrophils. Magnification = 160 x.

Fig. 22--Liver of an ovine fetus from a ewe inoculated intravenously with 10^6 *C.psittaci* and examined 56 days post-inoculation. Note the discrete focal accumulation of macrophages (granuloma) with abundant pale staining cytoplasm. Magnification = 160 x.

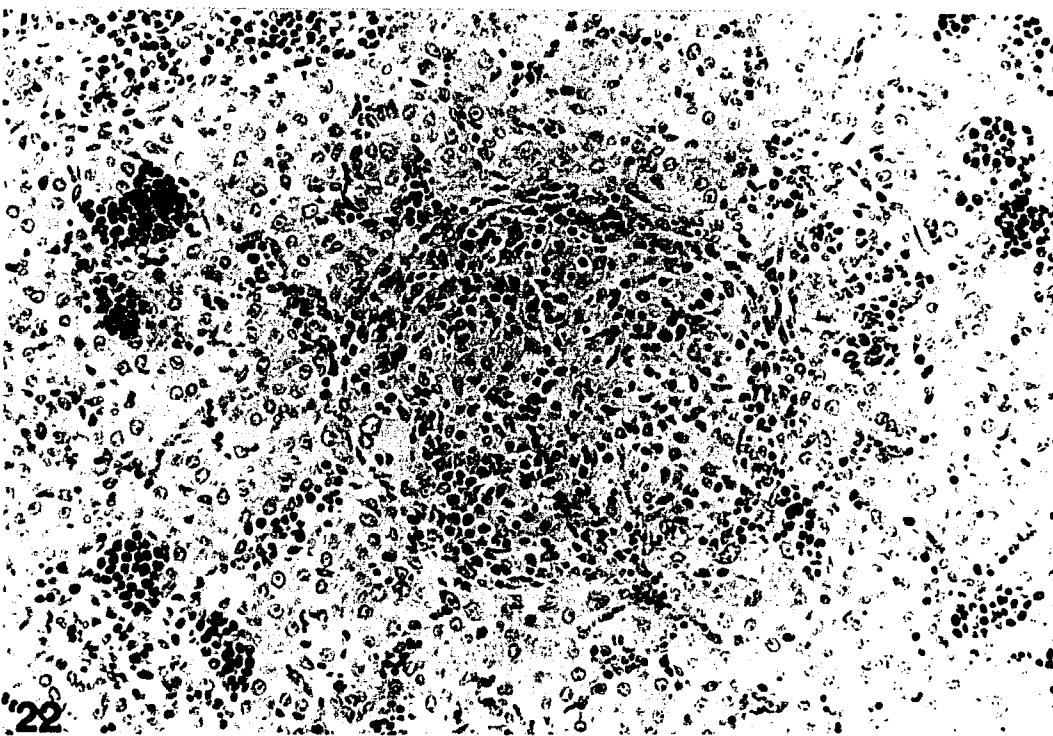
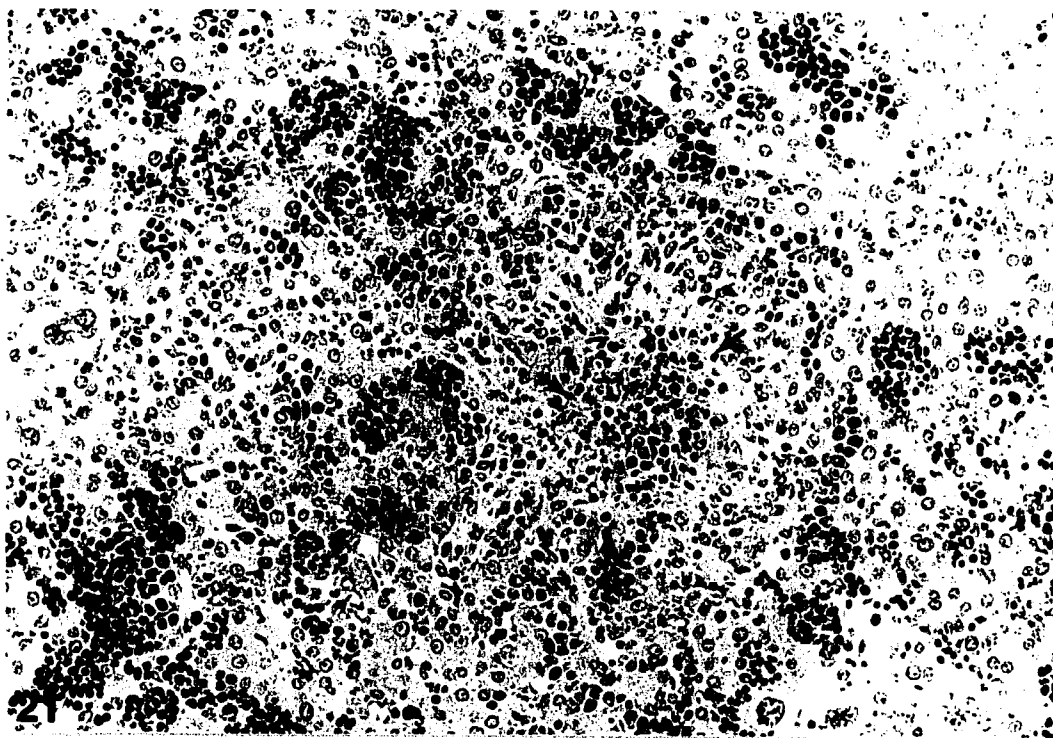


Fig. 23--Myocardium of an ovine fetus from a ewe inoculated intravenously with 10^6 *C.psittaci* and examined 49 days post-inoculation. Note the interstitial infiltrates of neutrophils and mononuclear cells. Magnification = 200 x.

Fig. 24--Spleen of an ovine from a ewe inoculated intravenously with 10^6 *C.psittaci* and examined 43 days post-inoculation. Note the small focal area of necrosis with associated neutrophilic infiltrates (arrows) and prominent lymphoid follicles. Magnification = 160 x.

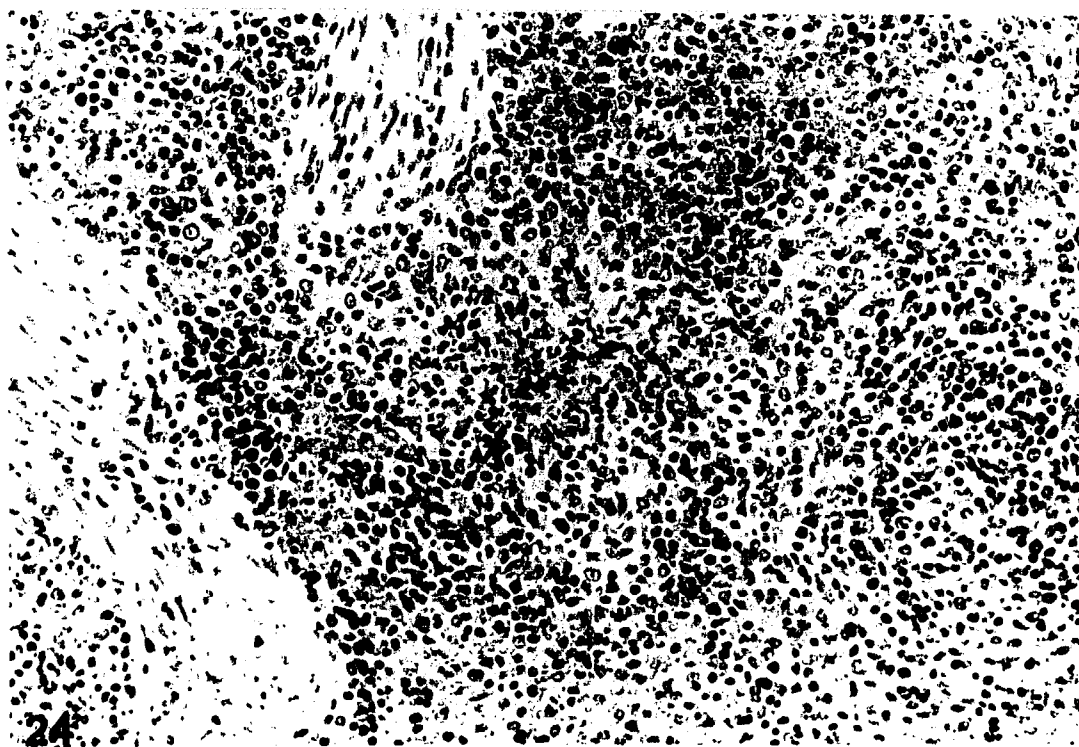
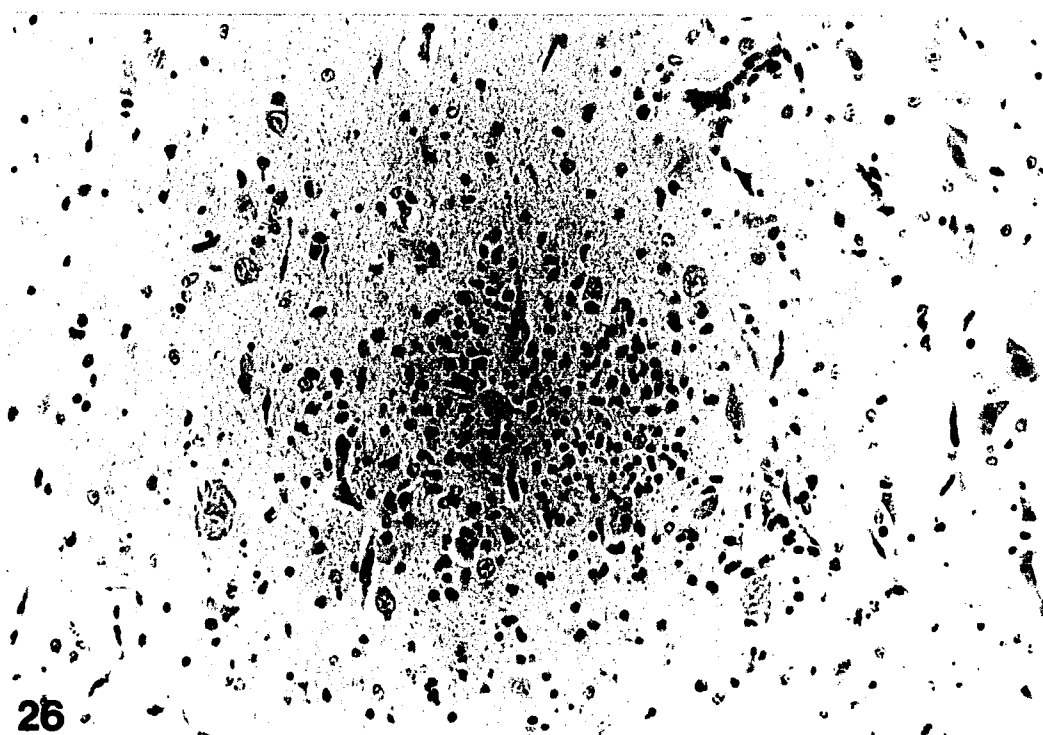
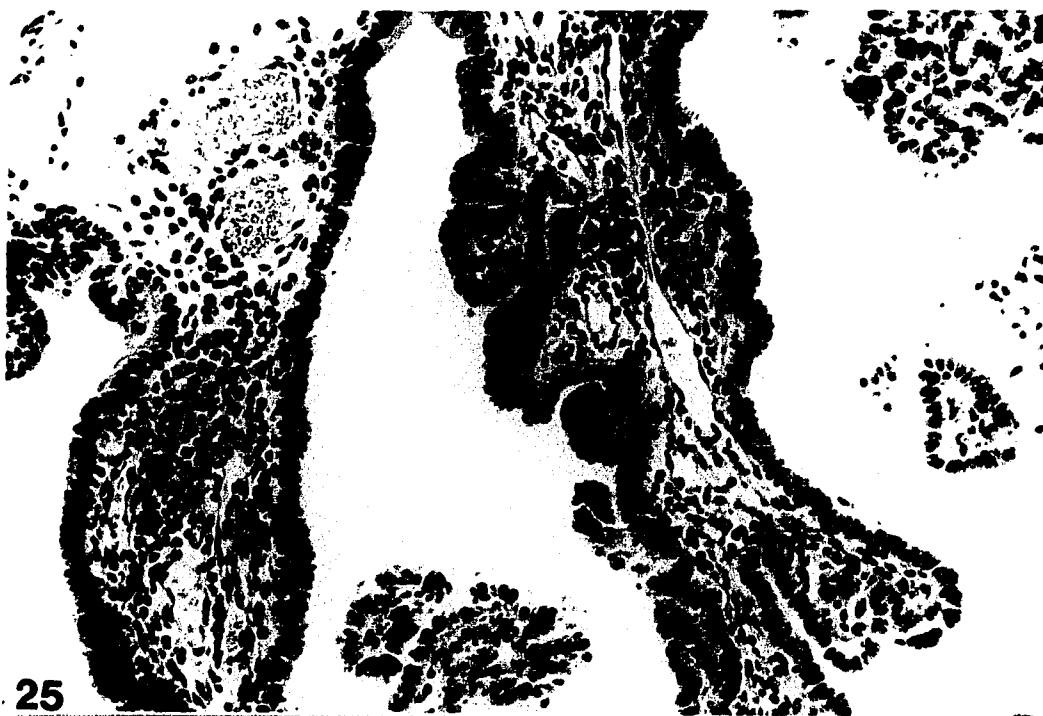


Fig. 25--Choroid plexus of an ovine fetus from a ewe inoculated intravenously with 10^6 *C.psittaci* and examined 47 days post-inoculation. Note the perivascular infiltrates of mononuclear cells, predominantly lymphocytes. Magnification = 160 x.

Fig. 26--Brain of an ovine fetus from a ewe inoculated intravenously with 10^6 *C.psittaci* and examined 47 days post-inoculation. Note the focal area of degeneration/necrosis and gliosis. Magnification = 200 x.



Discussion

Ewes in days 85 to 110 of gestation were susceptible to infection with *C. psittaci*. Placental and fetal infection was achieved in 22 of 40 susceptible ewes inoculated intravenously with *C. psittaci*. A suppurative placentitis was the predominant change associated with chlamydial infection. The gross and microscopic changes in the placentas were characteristic of those described for the natural disease and in other experimental studies.^{21,52, 240,253,271,306,334,349,352}

The distribution and progression of placental lesions suggested that intravenous inoculation of chlamydiae was followed by localization in erythrophagocytic chorionic trophoblasts lining placentomal hematomas. Hematomas develop around 60 days of gestation at the base of some chorionic villi and are composed of extravasated maternal blood that escapes from capillaries and larger blood vessels within tips of maternal septa.³⁹⁰ The hematomas provide an accessible route for chlamydiae from the maternal circulation to make direct contact with the chorionic epithelium.^{16,47,268,347} The well developed hematomas of the ovine placenta may explain why chlamydial abortions are more common in sheep than other species of food animals. Erythrophagocytic chorionic trophoblasts are actively engaged in the uptake and subsequent breakdown of maternal erythrocytes, probably as an important source of iron for the fetus. During chlamydemia, chlamydiae from the maternal blood stream probably are phagocytized by erythrophagocytic trophoblasts along with maternal erythrocytes. A similar mechanism has been demonstrated for other bacterial abortifacients including *Coxiella burnetti*,^{33,275} *Listeria monocytogenes*,²⁴⁸ *Brucella*

ovis,^{211,250} *Brucella melitensis*,²⁴⁷ *Brucella abortus*,^{17,249,292} and *Campylobacter fetus* var. *fetus* and *jejuni*.^{162,218} Recent studies have suggested that there may be a temporal restriction of chlamydial infection of the ovine placental hematomas until after 90 days of gestation.⁵⁰ The mechanisms are probably complex and may involve endocrinological and/or immune-related changes.

Although chlamydiae were isolated in low numbers from placentas collected 4 and 6 days post-inoculation, microscopic evidence of chlamydial infection was not identified until 14 days post inoculation (DPI). These early lesions consisted of chlamydial inclusions in erythrophagocytic chorionic trophoblasts lining placentomal hematomas. Recently, Buxton and colleagues in a study of chlamydial abortion, identified similar changes in ovine placentas examined 13 DPI.⁵⁰ From the placentomal hematomas chlamydiae rapidly spread to infect adjacent chorioallantoic trophoblasts of the peri- and interplacentome. Necrosis and ulceration of infected trophoblasts resulted in intense infiltrates of inflammatory cells, predominantly neutrophils. Accumulation of inflammatory exudate containing numerous chlamydiae in hematomas which are continuous with the uterine lumens probably facilitated the rapid spread of infection to the periplacentome and interplacentome. Inflammation of the periplacentome and interplacentome was first identified 21 DPI and 24 DPI, respectively. The severity and distribution of placental inflammation increased with the duration of the infection and the age of the fetus. Severe suppurative placentitis was a consistent finding in ewes infected with chlamydiae and examined 32-60 DPI. The endometrial epithelium did not appear to be highly important in the sequence of events in placental infection.

The progression of the infection *in utero* suggested that the fetus becomes infected hematogenously by chlamydial invasion of the fetal circulation. Following ulceration of the chorion, chlamydiae in the uterine lumens and placentomal hematomas are in direct contact with intraepithelial capillaries of the placenta. Intraepithelial capillaries and vessels in the chorionic stroma of the placenta drain into the chorioallantoic vessels, which are part of the fetal circulation. Oral, conjunctival, or respiratory infections of the fetus are possible and may play a lesser role. Amniotic and allantoic fluids likely become infected during the later stage of the disease, particularly when placental membranes rupture. Several studies have suggested that the ultimate pattern and progress of the disease in the fetus is similar, despite wide variations in the timing of maternal infection.^{268,332,349,350}

There were no diagnostic macroscopic lesions in chlamydiae-infected fetuses. Microscopic lesions were first identified in two fetuses examined 18 DPI, but were more frequent and extensive in chlamydiae infected fetuses examined 30-60 DPI. Lymphoid hyperplasia, infiltrates of mononuclear cells in portal areas of the liver, and multifocal hepatic necrosis were the most common histologic changes. These fetal lesions were consistent with other experimental studies and reports of natural disease.^{4,50,68,236,237,245,268,303,320,332,348-350} Although these lesions are not pathognomonic, their distribution and characteristics make them valuable diagnostically. Perhaps the more extensive lesions in older fetuses was due in part to their ability to generate a stronger inflammatory response. Studies have shown that in the ovine fetus neutrophil enzyme systems (esterase) are not present until 135 days of gestational age and monocyte systems (lipase) until after birth.³¹⁰

Lymphoid hyperplasia was a common finding in many late-term chlamydiae infected fetuses. Cortical and medullary development of these lymph nodes were indicative of both T and B cell stimulation. The increased immunoglobulin concentrations observed in fetuses infected with chlamydiae 24 or more days post inoculation also were consistent with immunologic stimulation and were in agreement with previous studies.¹⁶¹ Generally, the humoral immune response of late term ovine fetuses is believed to be comparable to neonates.^{119,310} A recent study demonstrated a significant increase in IgM- and IgG- positive cells in spleens and lymph nodes of ovine fetuses 25 days after infection with chlamydiae.⁵⁰ Additionally, the portal infiltrates of mononuclear cells and scattered accumulations of macrophages in fetal livers were suggestive of cell-mediated immune mechanisms. However, the role of the cellular immune system in the ovine fetal immune response is uncertain. Although the ability to reject allografts is present by mid-gestation and reticuloendothelial clearance is efficient in the newborn lamb, certain functions of the cellular immune response do not fully mature until after birth.³¹⁰

As expected, the placenta was the best specimen for isolation of chlamydiae. Placentas with characteristic gross and/or microscopic lesions usually contained high numbers of chlamydiae. In contrast, low numbers of chlamydiae were isolated from other fetal tissues. Chlamydial isolation was more successful from tissues of older fetuses examined 40-60 DPI. Conjunctival swabs, lung, spleen, amniotic fluids, and liver proved to be the best specimens for chlamydial isolation. Inactivation due to *in utero* autolysis and transport significantly reduce the numbers of viable chlamydiae in field specimens, but should not have been a

factor in this study. Conceivably, chlamydia-specific antibodies, which often were present in large amounts in late term chlamydiae-infected ovine fetuses, could interfere with chlamydial isolation procedures.

Eight ewes aborted 4 to 11 days after inoculation. These abortions were atypical, in that they did not resemble the naturally occurring disease. A similar "early abortion syndrome" associated with intravenous administration of chlamydiae has been previously described.³⁵⁰ The pathogenesis of these early abortions is uncertain, however, a chlamydiae-associated toxin has been implicated.³⁵⁰ This toxin may be chlamydial lipopolysaccharide (LPS), which is structurally and biochemically similar to that of other gram-negative organisms.^{41,57,57,269,270} Endotoxin induced abortions have been reported in mice, goats, pigs, cows, and horses.^{76,80-82,128,133} Recent studies in the horse have demonstrated decreased luteal activity with fetal loss after induced endotoxemia.⁸⁰⁻⁸² Neither endotoxin nor the clinical response during endotoxemia (i.e., fever, tachycardia, leukopenia) have a direct luteolytic or fetotoxic effect, but, rather, they act indirectly through the release of PGF_{2α} and the compromise of luteal activity.⁸¹ Early abortions associated with experimental infection with chlamydiae may have had a similar pathogenesis. The sporadic nature of these early abortions suggested that there may be individual variations in susceptibility.

In summary, my observations on the sequence of events in chlamydial placentitis suggested that chlamydiae localize in erythrophagocytic trophoblasts of placental hematomas and subsequently spread to other portions of the placentome and interplacentome. Intracellular replication in trophoblasts resulted in necrosis, ulceration, and an intense

suppurative inflammation. The placental lesions closely resembled the natural disease and were diagnostic. The fetus probably is infected hematogenously by chlamydial invasion of the fetal circulation. Lymphoid hyperplasia, multifocal hepatic necrosis, and infiltrates of mononuclear inflammatory cells in portal areas of the liver were the primary changes.

Elevated levels of IgG immunoglobulins were consistent findings in ovine fetuses infected with chlamydiae for ≥ 24 days. Chlamydial infection was confirmed by isolation procedures in tissue cell culture. High numbers of chlamydiae were isolated from infected placentas, whereas, other fetal tissues contained low numbers of viable organisms.

**4. IDENTIFICATION OF *CHLAMYDIA PSITTACI* IN
FORMALIN-FIXED, PARAFFIN-EMBEDDED OVINE
FETAL TISSUES WITH AN AVIDIN-BIOTIN-PEROXIDASE
COMPLEX (ABC) IMMUNOENZYMATIC STAINING TECHNIQUE**

Introduction

A diagnosis of chlamydial abortion (enzootic abortion of ewes) often is based upon the flock history, the presence of characteristic pathologic changes in the placenta, and demonstration of chlamydiae in placental smears or histologic sections.^{83,189,192,337} Isolation of chlamydiae may be difficult, especially under field conditions.^{178,189,192,245,337,339} Chlamydiae are readily inactivated in the course of intrauterine events that lead to fetal death.^{214,337,339,345} Contamination by other bacteria and unfavorable transport conditions further reduce the viability of chlamydiae.³³¹

Immunohistochemical methods, which have several variations including direct and indirect immunoperoxidase methods, peroxidase-antiperoxidase (PAP) techniques, and the avidin-biotin-peroxidase complex (ABC) procedures, have been used successfully to detect cellular antigens and infectious agents in various animal tissues.^{42,69,120,147-149,152,165,239,240,243,244,253,267,305,322,327,353,354,377} These staining procedures offer sensitive specific staining of tissue antigens and retention of tissue architecture.^{42,88,152,240} The ABC method has been shown to increase sensitivity of immunoperoxidase procedures by amplifying the antigen-antibody reaction.^{69,120,147,169,322}

Immunohistochemical methods for chlamydiae have been reported, but their availability and use have been limited.^{50,122,200} The objective of this study was to develop and evaluate an ABC method to detect chlamydial antigens in formalin-fixed paraffin-embedded tissues from ovine fetuses infected with

Materials and Methods

Experimental Design

Forty pregnant 6-18 month old ewes that were seronegative to chlamydiae by complement-fixation, received 10^6 inclusion-forming units of strain OSP of *C. psittaci* intravenously in days 85-110 of gestation. Sixty-three fetuses were collected at selected times from 4-60 days post-inoculation. Ten fetuses from 6 normal ewes also were examined and served as negative controls.

Each fetus was necropsied and appropriate tissues were collected for various diagnostic procedures as previously described.

Tissue Fixation and Processing

Sections of fetal lung, liver, kidney, spleen, heart, brain, lymph nodes, adrenal and placenta were fixed in 10% neutral buffered formalin. Fixation times varied from 24 hours to 8 months. Tissues were dehydrated in graded series of ethanol, cleared with xylene, processed in paraffin at 60° C, and embedded in paraffin. Sections were cut 4-5µm thick and were attached to glass slides precoated with poly-l-lysine. This adhesive ensures adherence of tissue sections to the slides.

Primary Antibodies

Polyclonal antiserum: Primary polyclonal antiserum was obtained from rabbits hyperimmunized with ovine abortion strains B577 and OSP of *C. psittaci*. Chlamydiae were grown in Vero cells and harvested when more than 20% of cells showed cytopathic effects. Vero cells were removed by low speed centrifugation and the supernatant was centrifuged at higher speed (30000 X g for 20 min). The supernatant was collected and elementary bodies (EB) were isolated by density gradient centrifugation in Renograffin (E.J. Squibb, NJ). The band containing EB was collected, suspended in 0.1 M phosphate-buffered saline (PBS)(pH 7.4) and centrifuged. The pellet was resuspended in 0.5% neutral buffered formalin in PBS and held for 12-18 hours at 4°C. Following centrifugation at 20000 x g for 15 minutes, the pellet was resuspended in PBS and emulsified 1:1 with complete and incomplete Freund's adjuvant (Sigma Chemical Co. St. Louis, MO).

Five 7-8 pound adult male New Zealand white rabbits that were seronegative to *C. psittaci* were employed. Chlamydial EB in PBS and complete Freund's adjuvant was used for the primary immunization and each rabbit received 1.0 ml (four 0.25 ml aliquots) in the quadriceps muscles. The first booster was given 2 weeks later in the neck and consisted of 1.0 ml (four 0.25 ml aliquots) of antigen in incomplete Freund's adjuvant (Sigma Chemical Co St. Louis, MO). Three weeks later the rabbits received a third booster immunization of 1.5 ml of 1:1 mixture of chlamydial EB in PBS in the interscapular subcutis. Serum samples were collected at 4 and 8 weeks and evaluated for chlamydial antibodies with the complement fixation test.(National Veterinary Services Laboratories, Ames, IA)

Two weeks after the third booster the rabbits were anesthetized with an intramuscular injections of ketamine (Bristol Laboratories, Bristol, TN) and acepromazine (Fort Dodge Laboratories, Ft. Dodge, IA) and were exsanguinated by cardiac puncture. Serum was collected, purified with CM Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) column chromatography, aliquoted and stored at -20°C. This chromatography procedure produces an IgG rich fraction with reduced albumin, protease, and serum complement proteins. Anti-chlamydiae antiserum was used as a primary antibody at a dilution of 1:750 in Tris-saline buffer (1 part 0.01 M Tris buffer to 9 parts 0.85% saline solution [pH 7.6]) containing 2.0% normal sheep serum. A dilution of 1:750 was chosen because of its reduced nonspecific binding to tissues. Pooled sera from normal rabbits, diluted 1:750 in Tris-saline buffer with 2.0% normal sheep serum, was used as negative control serum.

Monoclonal antibodies: Monoclonal antibodies (MAbs) to strain OSP of *C. psittaci* were produced as previously described.¹² Briefly, strain OSP was cultivated, purified, and used for mouse inoculation and production of MAbs. Monoclonal antibodies from ascites fluids were harvested from mice following intraperitoneal injection of hybridoma cells. Clones were selected for their ability to produce high levels of fluorescence with strain OSP in Vero cell culture. Sixteen clones were screened with an immunohistochemical procedure on sections of formalin-fixed ovine placenta infected with *C. psittaci*. A "cocktail" composed of equal volumes of 6 MAbs that produced the most intense immunohistochemical staining was purified with CM Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) column chromatography and was used as primary antibody at a dilution of 1:2500 in Tris-

saline containing 2% normal sheep serum. This working dilution was chosen because of its reduced nonspecific binding to tissues. Mouse ascites fluid, diluted 1:2500 in Tris-saline buffer with 2.0% normal sheep serum, was used as negative control serum.

Avidin Biotin Peroxidase Complex (ABC) Immunohistochemical Procedure

Deparaffinization and endogenous peroxidase blockage: Tissue sections were deparaffinized for 12 minutes with xylene and were hydrated for 20 minutes in a graded series of ethanol containing 0.1% concentrated hydrochloric acid. The sections were washed twice for 5 minutes each in 0.01 M Tris buffer (pH 7.6) containing .001% Tween 20 (Tris-tween). Endogenous enzyme activity was blocked by treatment with 3% hydrogen peroxide in methanol for 15 minutes. This treatment was followed by two 5 minute washes in Tris-tween buffer. All steps were conducted at room temperature (25° C).

Proteolytic enzyme digestion and blockage of non-specific binding sites:

Tissue sections were placed in Tris-tween buffer at 37°C for 5 minutes followed by 5 minutes in Tris buffer containing 0.1% trypsin and .1% calcium chloride at 37°C, and two washes in Tris-tween buffer.⁽⁷⁸⁾ Nonspecific immunoglobulin adherence was minimized (blocking) by treatment of tissues with 20% normal horse serum in Tris buffer for 30 minutes in a humidity chamber at room temperature (25° C)

Staining procedure: Normal horse serum was removed from each tissue section by inverting the slide and blotting to remove excess fluid. Primary polyclonal antiserum or monoclonal antibodies were applied to tissue sections overnight in a humidity chamber at 4°C. Tissue sections were rinsed with buffer, washed for 10 minutes in Tris-tween buffer,

and excess fluid was removed by blotting. Depending upon the primary antibody that was used, tissue sections were covered with either biotinylated horse anti-mouse or anti-rabbit IgG (heavy- and light-chain specific) (Vector Laboratories, Burlingame, CA) diluted to 1:200 in Tris-saline buffer for 30 minutes at 37°C. After washing for 10 minutes in Tris-tween buffer, tissue sections were incubated with horseradish peroxidase streptavidin (Vector Laboratories, Burlingame, CA) diluted to 1:200 in Tris-saline buffer for 45-60 minutes in a humidity chamber at room temperature (25°C). Tissue sections were washed for 10 minutes and then covered with peroxidase substrate-chromogen solution.

Substrate-chromogen solution and counterstain: The substrate-chromogen solution was freshly prepared before use. The 3,3 diaminobenzidine (DAB) (Sigma Chemical Co. St. Louis, MO) was diluted to 0.06% in Tris buffer (pH 7.6) containing 0.03% H₂O₂. Tissue sections were flooded with substrate chromogen solution and incubated for 3-5 minutes at room temperature. Sections were washed with ultra-pure water for 10-15 minutes, counterstained with Gills hematoxylin, and dehydrated with graded ethanol baths and xylene. Coverslips were mounted with water-insoluble medium. Oxidized DAB forms a brown precipitate at the site of peroxidase localization in the tissue section.

Controls: The specificity of the anti-chlamydiae primary antibodies was assessed by evaluation of sections of each tissue with either mouse ascites fluid or normal rabbit serum as primary antibody and sections of normal ovine placentas and fetuses with anti-chlamydiae primary antibodies. Additionally, tissues were collected from animals with other bacterial diseases and were processed with this immunohistochemical technique. Bacteria in these

tissues were visible with light microscopic examination of hematoxylin and eosin stained sections and were identified by culture techniques. Bacteria evaluated included *Listeria monocytogenes* in fetal bovine spleen, *Bacillus cereus* in bovine placenta, *Campylobacter jejuni* in an ovine placenta, *Brucella abortus* in caprine placenta, and *Pasteurella multocida*, *Actinomyces pyogenes*, and *Actinobacillus pleuropneumoniae* in porcine lungs.

Results

This avidin-biotin-peroxidase complex immunohistochemical procedure (ABC) using either mouse monoclonal antibodies or rabbit polyclonal antisera, clearly and specifically labelled chlamydial antigen in ovine placentas. Dark brown DAB precipitate identified the presence of chlamydial antigen. Chlamydial antigen was identified in 36 of 38 placentas from which chlamydiae were isolated (Table 7). Chorionic trophoblasts often were packed with brown staining precipitate and intracytoplasmic staining often obscured cell detail (Figs 28-32). Exudate in placentomal hematomas and uterine spaces contained abundant chlamydial antigen (Figs. 27,28,32,33). Chlamydial antigen was not identified in leukocytes, placental mesenchymal tissues (stroma), and blood vessels. When placental inflammation was severe, chlamydial antigen was present multifocally in endometrial epithelial cells (Fig. 34) and in exudate on the luminal surface of the endometrium.

All placentas in which chlamydial antigen was identified with this ABC method, had gross and/or microscopic lesions that were characteristic of infection with chlamydiae. In addition, high numbers of chlamydiae were isolated from each of these placentas. Chlamydial antigen was not detected in two chlamydiae-infected placentas from ewes

examined 4 and 6 days post-inoculation. These specimens contained low numbers of chlamydiae and there were no gross or microscopic changes.

This ABC procedure did not consistently identify chlamydial antigen in other fetal tissues (Table 7). Immunohistochemical staining consisted of small collections of mononuclear cells and/or macrophages with pale yellow to dark brown cytoplasm in some lymph nodes and spleens (Fig 35). These cells were not identified in control tissues and staining was not present when sections were stained with normal rabbit sera or mouse ascitic fluids. However, no chlamydial inclusions or extracellular chlamydiae were identified in these tissues with this immunostaining method. Surprisingly, chlamydial antigen was not associated with necrotic foci in the liver and spleen.

Chlamydiae did not stain with this ABC method when normal rabbit serum and mouse ascitic fluids (Fig 36) were used as primary antibodies. Other tests for specificity, which included reacting chlamydial antibodies with uninfected tissues and control (chlamydiae negative) antibodies with antigen-containing tissues, showed no evidence of nonspecific reactivity. Additionally, there was no significant staining of the other bacteria that were evaluated (Fig. 37). There was minimal non-specific (background) staining. However, there was slightly more nonspecific staining with the rabbit antiserum, but it did not interfere with identification of chlamydial antigen. The intensity of specific staining was slightly reduced and background staining was increased in severely autolyzed ovine placental tissues. (Personal observation) Staining intensity did not vary significantly among tissues fixed up to 8 months. Brown pigmented substances, which were presumed to be

blood pigments, were in various infected and non-infected tissues. These pigments often were more abundant in the livers and stained with primary antibodies, mouse ascitic fluids, and normal rabbit serum.

Table 7. Comparison of chlamydial isolation in cell culture with avidin-biotin-peroxidase complex (ABC) immunostaining for detection of chlamydial antigen in formalin-fixed and paraffin embedded tissues from ovine fetuses exposed to *Chlamydia psittaci*

<i>C. psittaci</i> Isolation	No. of tissues ^a	
	Positive ABC	Examined
Placenta		
Positive	36	38
Negative	0	26
Liver		
Positive	0	11
Negative	0	53
Lung		
Positive	0	12
Negative	0	52
Kidney		
Positive	0	9
Negative	0	55
Spleen		
Positive	5	14
Negative	7	50
Lymph nodes		
Positive	5	10
Negative	2	54
Other tissues ^b		
Positive	0	0
Negative	0	320

^a Tissues from 64 ovine fetuses from 40 ewes experimentally inoculated with *C. psittaci*.

^b Myocardium, intestines, brain, thymus, and thyroids from 64 fetuses.

Fig. 27--Placentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on day 43 post-inoculation. Note diffuse ulceration of chorionic trophoblasts lining placentomal hematoma, inflammation of subepithelial mesenchymal tissues (stroma), and abundant exudate in hematomas. Hematoxylin & Eosin. Magnification = 100 x.

Fig. 28--Placentomal hematoma from Fig. 1. Brown peroxidase reaction product signifies location of abundant chlamydial antigen in hematoma. Tissues were stained with an avidin-biotin-peroxidase complex technique, using hematoxylin counterstain. Murine monoclonal antibodies were used as primary antibody. Magnification = 100 x.

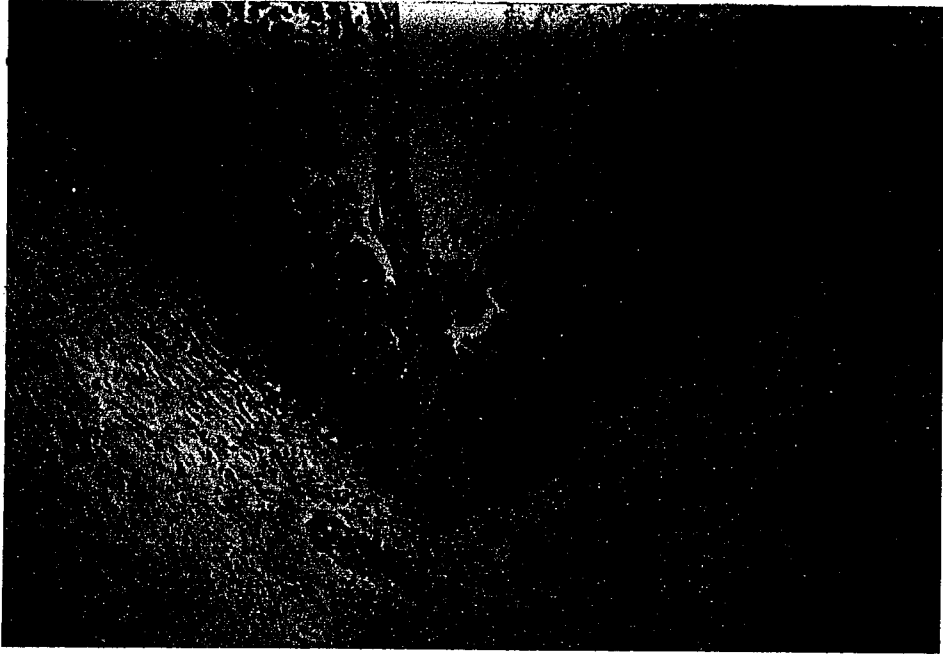


Fig. 29--Chorioallantoic membrane from the interplacentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on day 43 post-inoculation. Brown peroxidase reaction product signifies location of chlamydial antigen in chorioallantoic trophoblasts. Tissues were stained with an avidin-biotin-peroxidase complex technique, using hematoxylin counterstain. Murine monoclonal antibodies were used as primary antibody. Magnification = 200 x.

Fig. 30--Chorioallantoic membrane from Fig. 29. Brown DAB signifies location of chlamydial antigen in chorioallantoic trophoblasts. Note brown DAB precipitate obscures cell detail. Tissues were stained with an avidin-biotin-peroxidase complex technique, using hematoxylin counterstain. Murine monoclonal antibodies were used as primary antibody. Magnification = 600 x.

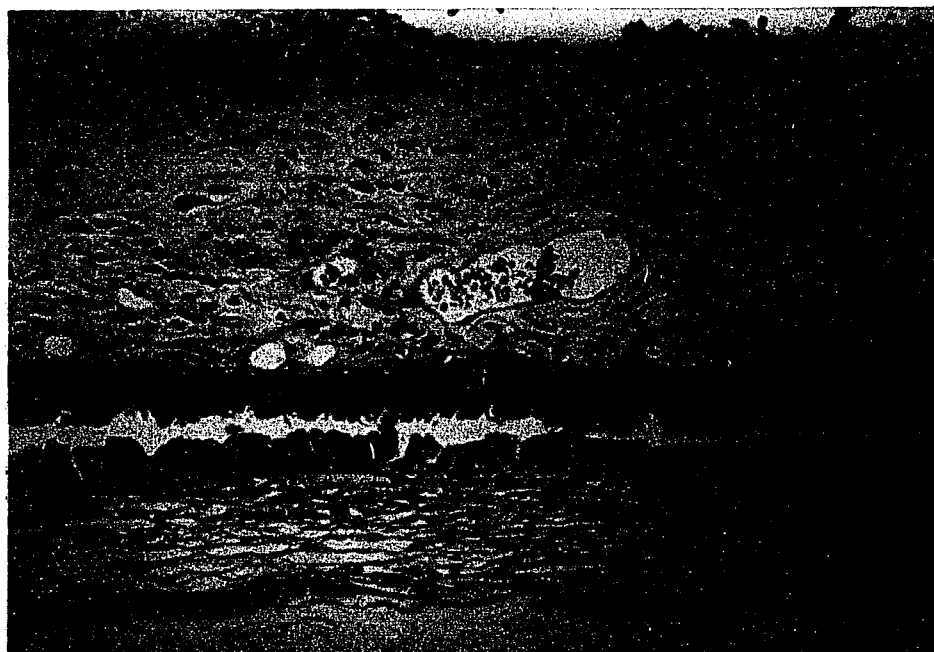


Fig. 31--Chorionic villus from a placentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on day 39 post-inoculation. Brown peroxidase reaction product signifies location of chlamydial antigen in cytoplasm of chorionic trophoblasts. Tissues were stained with an avidin-biotin-peroxidase complex technique, using hematoxylin counterstain. Murine monoclonal antibodies were used as primary antibody. Magnification = 400 x.

Fig. 32--Placentome from Fig. 5. Brown peroxidase reaction product signifies location of chlamydial antigen in desquamated degenerate trophoblasts between chorionic villi. Tissues were stained with an avidin-biotin-peroxidase complex technique, using hematoxylin counterstain. Murine monoclonal antibodies were used as primary antibody. Magnification = 200 x.

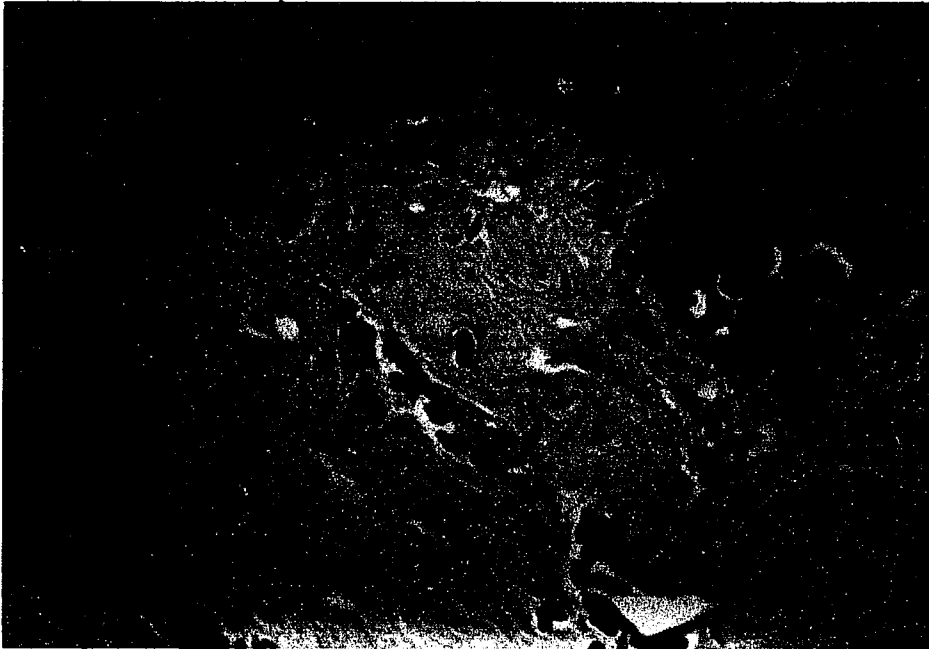


Fig. 33--Chorioallantoic membrane from interplacentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined on day 58 post-inoculation. Brown peroxidase reaction product signifies location of chlamydial antigen on surface of ulcerated chorion. Rabbit anti-chlamydia serum was used as primary antibody. Tissues were stained with an avidin-biotin-peroxidase complex technique, using hematoxylin counterstain. Magnification = 100 x.

Fig. 34--Endometrium of a ewe inoculated intravenously with intravenously with 10^6 *C. psittaci* and examined on day 43 post-inoculation. Brown peroxidase reaction product signifies location of chlamydial antigen in endometrial epithelial cells. Tissues were stained with an avidin-biotin-peroxidase complex technique, using hematoxylin counterstain. Murine monoclonal antibodies were used as primary antibody. Magnification = 400 x.

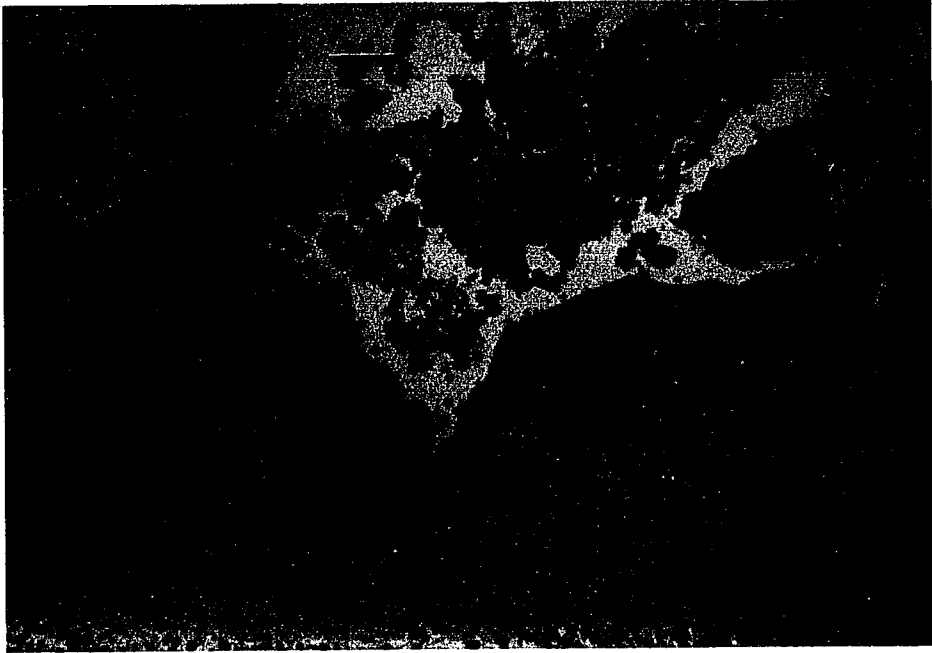


Fig 35--Placentomal hematoma from Figs. 1,2. Mouse ascitic fluid was used as primary antibody. Note that chlamydial antigen in hematomas did not stain with this avidin-biotin-peroxidase complex technique. Magnification = 200 x.

Fig. 36--Spleen of an ovine fetus examined 43 days after inoculation of the ewe with 10^6 *C. psittaci*. Scattered mononuclear cells with brown-staining cytoplasm. Tissues were stained with an avidin-biotin-peroxidase complex technique, using hematoxylin counterstain. Murine monoclonal antibodies were used as primary antibody. Magnification = 400 x.

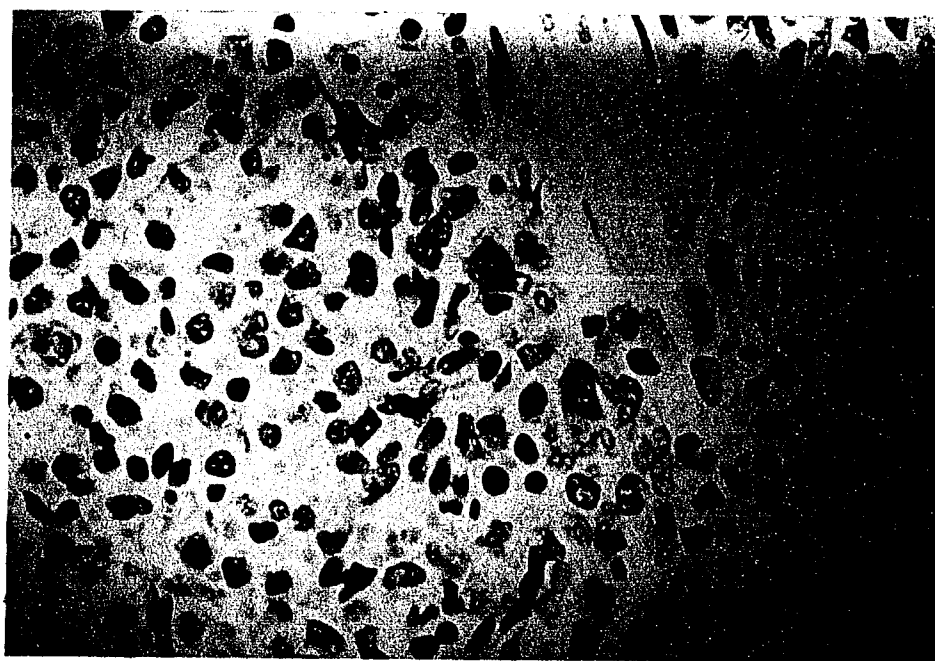
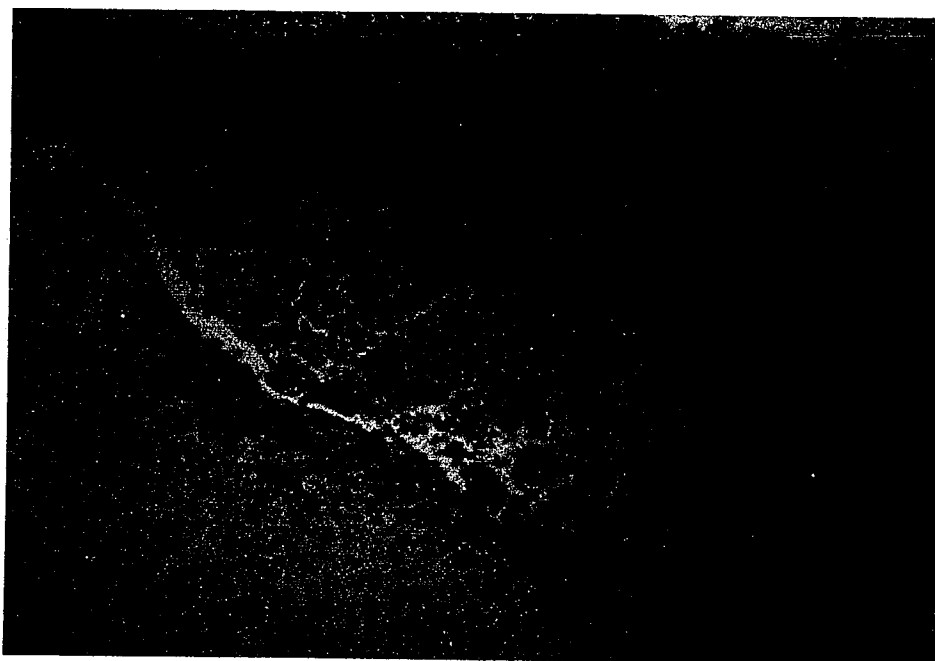
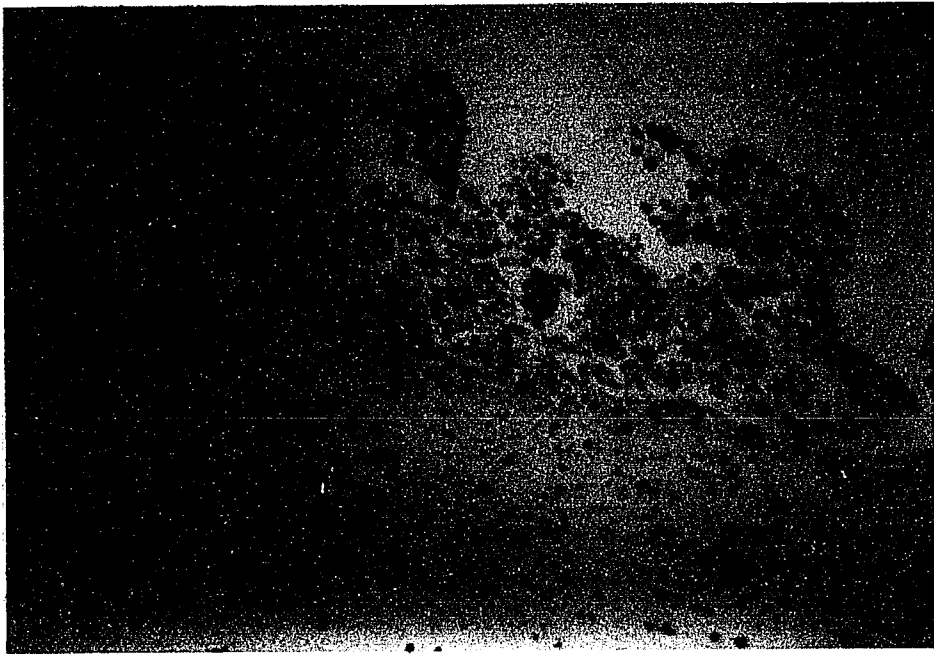


Fig. 37--Chorioallantoic membrane from the interplacentome of a goat infected with *Brucella abortus*. The chorion is diffusely ulcerated and abundant exudate containing numerous brucellae is present on the luminal surface (uterine space). Note the absence of staining with this avidin-biotin-peroxidase complex technique. Murine monoclonal anti-chlamydial antibodies were used as primary antibody. Magnification = 200 x.



Discussion

This avidin-biotin-complex immunoperoxidase (ABC) staining technique clearly identified chlamydial antigen in formalin-fixed paraffin embedded ovine placental tissues. Chlamydial antigen was identified in all placentas with gross and microscopic lesions characteristic of chlamydial placentitis. The 2 chlamydiae-infected placentas from which chlamydial antigen was not identified were collected 4 and 6 days after inoculation of the ewe; there was no evidence of placental inflammation and low numbers of chlamydiae were isolated from each. Perhaps the amount of chlamydial antigen in these two placentas were less than could be detected with this immunohistochemical method. Additionally, in chlamydiae-infected placentas without macroscopic lesions, random histologic sections may have missed areas of inflammation.

This ABC procedure proved insensitive for detection of chlamydial antigen in other fetal tissues. No chlamydial antigen was detected in sections of most fetal tissues from which chlamydiae were isolated. Immunohistochemical staining in fetal tissues was limited to scattered macrophages and mononuclear cells in lymph nodes and spleens. Since no chlamydial inclusions were identified in fetal tissues it is uncertain whether the material in these cells represented phagocytized chlamydial antigen. Other studies using immunohistochemical procedures in formalin-fixed avian, canine, and feline tissues infected with chlamydiae, have reported similar findings and also have questioned their significance.^{252,253} In a recent study, Buxton and coworkers using a similar immunoperoxidase procedure on formalin fixed tissues from ovine fetuses infected with *C.*

psittaci, rarely identified small amounts of chlamydial antigen in fetal tissues.^{50,183}

Seemingly, the amount of chlamydial antigen in these fetal tissues was less than could be detected with this ABC method. Meador and colleagues determined that a minimum of 10^6 *Brucella abortus*/gram of tissue was needed for detection with a similar ABC immunohistochemical procedure.²⁴⁰ Although the minimum concentration of organisms/antigen in tissue necessary for detection with an immunohistochemical method will vary between species and tissues, possibly the limitations for detection of *C. psittaci* with this ABC method are similar. Surprisingly, no chlamydial antigen was identified in necrotic foci in fetal livers and spleens. Although it was likely that these necrotic foci were caused by direct cell injury due to chlamydial replication, tissue damage due to bacterial toxins and/or inflammatory mediators absorbed from the placenta cannot be rule-out.

The detection of antigen in tissues that had been held in formalin for 8 months indicated that the chlamydial antigen was not adversely affected by prolonged tissue fixation. Although staining intensity was slightly reduced, chlamydial antigen was clearly demonstrated in autolyzed placentas. Excessive nonspecific staining (background) was not a significant problem with this ABC procedure. Background staining was minimized by using relatively high dilutions of primary antibodies and overnight incubation at 4°C. It is possible to reduce the time required for incubation with primary antibody by reducing the dilution of antibody and increasing the incubation temperature. However, both changes increase the potential for nonspecific staining. Certain tissues, particularly livers and spleens, often contained abundant brown pigment (presumed to be blood pigments) that was

difficult to differentiate from DAB precipitates.

The results of this study suggested that this immunohistochemical procedure was specific for chlamydiae. There was no staining in tissues from normal control fetuses. Additionally, there was minimal staining of other bacteria. Cross-reactions with certain other gram-negative bacteria has been a problem with many immunologic procedures for chlamydiae, especially those using polyclonal antisera. Shared antigenic domains of lipopolysaccharide (LPS) is considered the basis of these cross-reactions.^{41,57,57,269,270} However, even if cross-reactivity does occur, the morphology of chlamydiae usually is distinctly different from other bacteria.

In summary, this immunohistochemical procedure was useful for detection of chlamydial infections in ovine placentas. Chlamydial isolation procedures were more sensitive for detecting *C. psittaci* than this ABC method, particularly with fetal tissues. Chlamydial antigen is relatively stable and withstands autolysis.

5. AN ULTRASTRUCTURAL STUDY OF CHLAMYDIAL PLACENTITIS

Introduction

Chlamydiae are obligate intracellular parasites that possess a distinctive developmental cycle and are responsible for numerous disease syndromes in animals including abortions in sheep. Infection of susceptible pregnant ewes with abortion strains of *Chlamydia psittaci* may cause abortion, stillbirths, or weak lambs.^{219,237,323,341} The gross and microscopic lesions of chlamydial placentitis are well described.^{4,50,268,337}

The distribution and progression of placental lesions suggests that *C. psittaci* initially infects erythrophagocytic trophoblasts of the placentomal hematomas with subsequent spread to chorioallantoic trophoblasts of the peri- and interplacentome.^{50,268,337} A similar mechanism has been demonstrated for other bacterial abortifacients of sheep including *Coxiella burnetti*,^{33,275} *Listeria monocytogenes*,²⁴⁸ *Brucella ovis*,^{211,250} *Brucella melitensis*,²⁴⁷ *Brucella abortus*,^{17,249,292} and *Campylobacter fetus* var. *fetus* and *jejuni*.^{162,218} Since chlamydiae have a unique developmental cycle, infected cells often can be identified ultrastructurally. The purpose of this study was to evaluate the ultrastructural changes of the ovine placenta associated with infection of *C. psittaci*. The initial site of chlamydial infection was my primary interest.

Materials and Methods

Experimental Design

Forty pregnant primiparous ewes that were seronegative to *C. psittaci* by complement fixation, received 10^6 inclusion-forming units of strain OSP an abortion strain of *C. psittaci* intravenously in days 85-110 of gestation. Sixty-four fetuses were collected at selected times from 4-60 days post-inoculation. Ten fetuses from 6 normal ewes also were examined and served as controls. Necropsy and uterine perfusion techniques were described previously.

Tissue Preparation

Samples of fixed placentome and intercotyledonary placenta were selected from perfused uteri and minced into 1 mm³ blocks for immersion fixation in the glutaraldehyde-paraformaldehyde mixture at 4° C. After 2 hours of fixation, tissue samples were rinsed in cacodylate buffer, stained *en bloc* in 1% osmium tetroxide, dehydrated in alcohols, cleared in propylene oxide, and embedded in epoxy resin.

Samples of selected placentomes were minced, immersion fixed in freshly depolymerized 4% paraformaldehyde in phosphate buffer, (pH 7.2) at 4°C for 2-4 hours followed by storage in 70% ethyl alcohol at 4°C. These tissue samples were washed, dehydrated in a graded ethanol series, and embedded in medium grade LR White (The London Resin Co. Ltd., Woking, Surrey, GU21 1AE, England), a polyhydroxy aromatic hydrophilic acrylic resin.¹¹³

Electron Microscopy

All sections of tissue were cut at 1 μm , stained with toluidine blue, and examined with light microscopy. Ultrathin sections from selected areas were cut, stained with 2% uranyl acetate and 2.5% lead citrate, and examined with a Phillips 410 transmission electron microscope.

Immunohistochemistry

Immunogold labelling of *C. psittaci* was performed on thin sections of LR white resin-embedded tissues mounted on nickel grids using post-embedding gold labelling techniques (Personal communication, Dr. J.T. Tappe, National Animal Disease Center) Each grid was washed with 25 ml of boiled ultrapure water. Washed grids were floated in 1.0% ovalbumin (OA) (Sigma Chemical Co., St. Louis, MO) in Tris buffer (pH 8.0) at 37°C for 60 minutes to block nonspecific binding sites. Following blotting to remove excess blocker, grids were placed in 0.1% OA in Tris buffer (pH 8.0) containing a mixture of equal volumes of 6 monoclonal antibodies to strain OSP of *C. psittaci* diluted 1/1000, and incubated for 18 hours at 4° C. Grids were washed in Tris buffer containing 0.1% OA and were placed in a 1:10 solution of gold labelled goat anti-mouse antibody (Auroprobe EM GAM IgG G10- Amersham Corporation, Arlington Heights, IL) in 0.1% OA in Tris buffer for 2 hours at 37° C. Grids were jet-washed (total of 25 ml) with Tris buffer containing 0.1% OA followed by washing in boiled, distilled water, blotted dry and stained with 2% uranyl acetate and 2.5% lead citrate. Sections of non-infected tissues and grids from which the primary antibody was omitted from the labelling procedure were used as controls.

Results

Chlamydiae were located in membrane bound inclusions (endosomes) in the cytoplasm of chorionic trophoblasts and free in placental hematomas and uterine spaces. Depending on the stage of the developmental cycle, these membrane bound inclusions ranged from small structures in the perinuclear cytoplasm that contained few organisms to those with numerous chlamydial bodies that occupied most of the cytoplasm and displaced organelles. The composition of the inclusions varied with the stage of the chlamydial developmental cycle. In early stages, inclusions contained primarily reticulate bodies (RB) the non-infectious vegetative forms of chlamydiae. Reticulate bodies, ranged from 0.5 to 1.3 μm in diameter and consisted of homogenous to granular (ribosomes) cytoplasm surrounded by two trilaminar membranes (cell wall and cytoplasmic membranes) (Figs. 38,39). Some RB showed the process of binary fission (Figs. 38,39). In later stages, inclusions contained numerous elementary bodies (EB) with fewer RB. Elementary bodies, the infectious form of chlamydiae, were more dense and ranged from 0.3 to 0.5 μm in diameter. EB often contained a dense eccentric nucleus (nucleoid) that was in contact with the cell wall. The cytoplasm was composed of granular to moderately dense amorphous material. Condensing forms, also known as intermediate bodies (IB) which ranged from 0.5-1.0 μm in diameter and had a distinct dense central accumulation of nuclear material (nucleoid) and granular cytoplasm also were numerous in later stages of development (Fig. 39).

The changes in infected chorionic trophoblasts varied with the stage of chlamydial

development. Minimal or no cellular degeneration was noted in early stages of chlamydial development (Fig. 40). Whereas, in advanced stages of the developmental cycle, chlamydial inclusions occupied most of the cytoplasm and displaced organelles to the periphery and the nucleus to basilar areas. Cell swelling, degranulation of ribosomes, dilation and vesiculation of endoplasmic reticulum, loss of organelles particularly mitochondria, chromatolysis, and loss of microvilli were common cellular changes in later stages of development (Fig. 41). Ballooning of cells resulted in rupture of plasma and inclusion membranes and chlamydiae were released into hematomas and uterine spaces. Additionally, many degenerate trophoblasts filled with chlamydiae sloughed into hematomas or uterine spaces (Fig. 42).

Chlamydiae were first identified in erythrophagocytic trophoblasts of a placentome from a ewe examined 14 days post-inoculation (DPI). Scattered erythrophagocytic trophoblasts lining placentomal hematomas contained membrane bound chlamydial inclusions in the perinuclear cytoplasm (Figs.43,44). No abnormalities were identified in basement membranes, fetal capillaries, or subepithelial mesenchymal tissues (stroma). By 18 DPI, many erythrophagocytic chorionic trophoblasts lining placentomal hematomas contained chlamydial inclusions (Fig. 45). Multifocally, trophoblasts were necrotic and/or had sloughed into the hematoma and subepithelial mesenchymal tissues were edematous and contained mild infiltrates of neutrophils and macrophages (Fig. 46). Hematomas contained free chlamydiae mixed with sloughed degenerate chlamydiae-infected trophoblasts and cell debris (Fig.47). By 28 DPI, there was extensive necrosis and ulceration of chorionic

trophoblasts lining placental hematomas(Figs.48,49). Necrosis and ulceration of trophoblasts and/or damage to basement membranes resulted in edema and intense infiltrates of inflammatory cells, predominantly neutrophils, in the subepithelial mesenchymal tissues (stroma)(Fig. 50). In areas of intense inflammation the endothelium of fetal capillaries was swollen, necrotic, and/or separated from the basement membrane. Occasionally, accumulations of cell debris and degenerate leukocytes were present in capillary lumens. Placental hematomas contained degenerate chlamydiae-infected chorionic trophoblasts, numerous free chlamydiae, cell debris, and neutrophils. Additionally, necrosis of chorionic trophoblasts of chorionic villi and maternal epithelium (syncytium) was present in placentomes examined ≥ 28 DPI. The mesenchymal tissues (stroma) of chorionic villi were edematous and infiltrated with leukocytes, predominantly neutrophils. Cell debris, chlamydiae, and leukocytes were present in intervillous spaces. Similar, although more extensive changes were present in chlamydiae-infected placentomes examined 30-60 DPI.

Chlamydial infection of chorioallantoic trophoblasts of the periplacentome were first identified in a placenta examined 21 DPI (Fig. 51) and the interplacentome at 24 DPI. Chlamydiae-infected erythrophagocytic trophoblasts at the edges of placentomes were continuous with infected chorioallantoic trophoblasts of the periplacentome. A similar progression of events-chlamydial infection and replication in chorioallantoic trophoblasts, necrosis and/or sloughing of infected trophoblasts, edema, and infiltrates of neutrophils-were observed. Extensive necrosis and ulceration of chorioallantoic trophoblasts of the interplacentome was present by 28 DPI. Inflammation resulted in separation of maternal

from fetal epithelium and accumulations of exudate composed of necrotic cell debris, degenerate chlamydiae-infected trophoblasts, free chlamydiae, and leukocytes on the ulcerated chorioallantoic membrane and in uterine spaces (Fig. 52). The subepithelial mesenchymal tissues (stroma) were markedly edematous and contained numerous neutrophils, fewer macrophages, and varying amounts of cell debris. The endothelium of fetal capillaries was necrotic or absent and capillary lumens contained cell debris (Fig. 52). Additionally, when placental inflammation was severe, maternal epithelium was necrotic and/or ulcerated. Infiltrates of neutrophils, macrophages, and lymphocytes were present in the propria/submucosa of the endometrium.

Unfortunately, the cellular detail in tissues preserved with paraformaldehyde fixation and embedded in LR white resin was relatively poor. Sectioning of these tissues was difficult and there were numerous artifacts. Additionally, tissue sections were unstable in the electron beam. Nevertheless, some sections were adequate for identification of chlamydiae with this immunogold labelling method. Immunogold particles attached to outer membranes and less often overlaid the cytoplasm of all chlamydial forms (Fig. 53). Chlamydiae in trophoblasts and free in hematomas/uterine spaces were stained with this procedure. Chlamydial antigen was not identified in placental mesenchymal tissues (stroma), leukocytes, and endothelium of capillaries. No gold labelling was present in either chlamydiae-infected placental sections treated with mouse ascitic fluids or normal placental sections treated with this procedure.

Fig. 38--Placentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined 24 days post inoculation. Erythrophagocytic chorionic trophoblasts from placentomal hematoma contain membrane bound chlamydial inclusions. These inclusions contain all chlamydial forms- reticulate bodies (RB), elementary bodies (EB), and intermediate bodies (IB). Many reticulate bodies are in binary fission. Magnification = 13400 x.

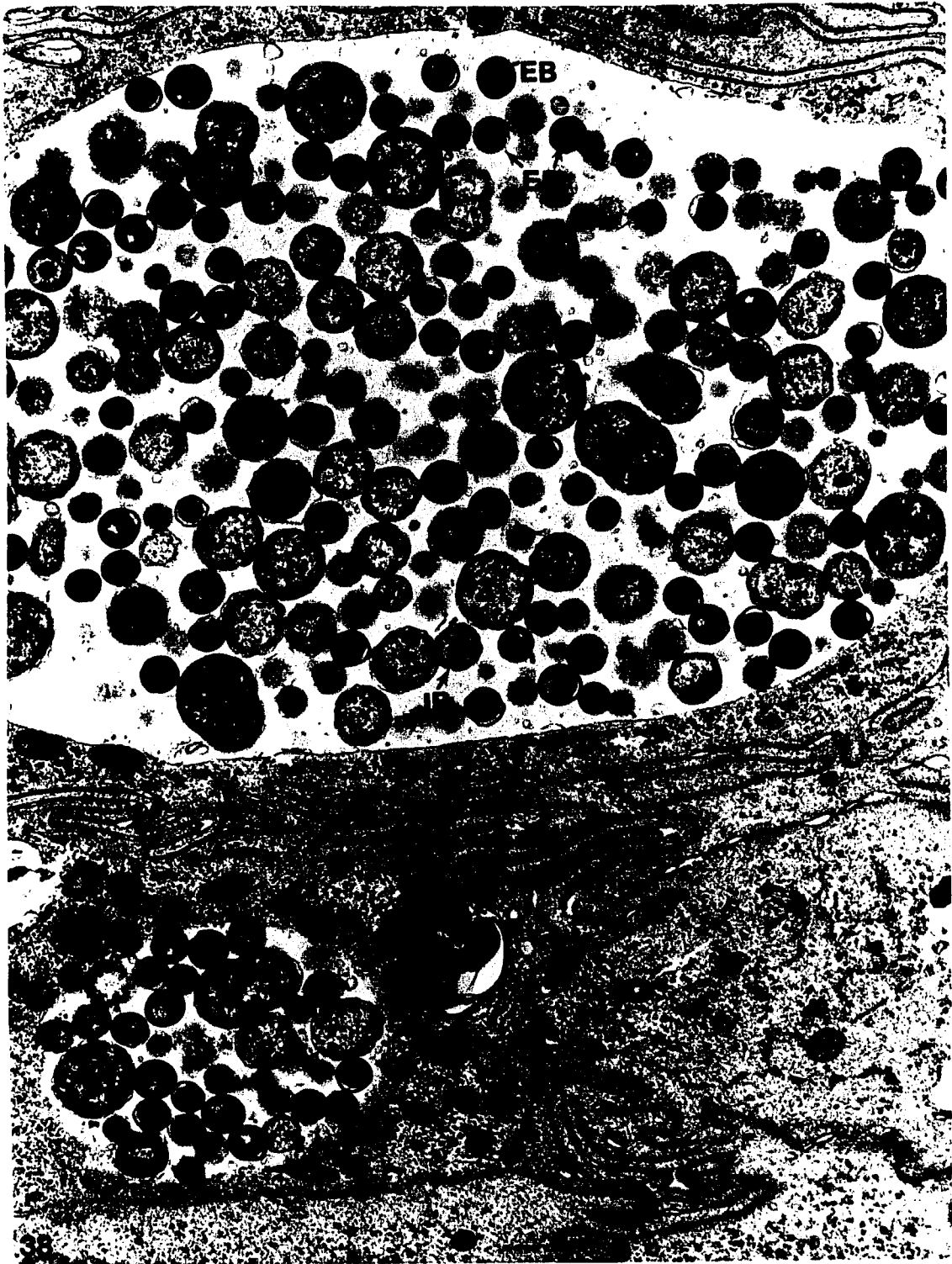


Fig. 39--Chlamydial inclusion from Fig. 38. Reticulate bodies (RB), intermediate bodies (IB), and elementary bodies (EB). Note two distinct membranes (cell wall and plasma membrane) surrounding the reticulate bodies and reticulate bodies in binary fission.

Magnification = 40000 x. Bar = 0.3 μ m

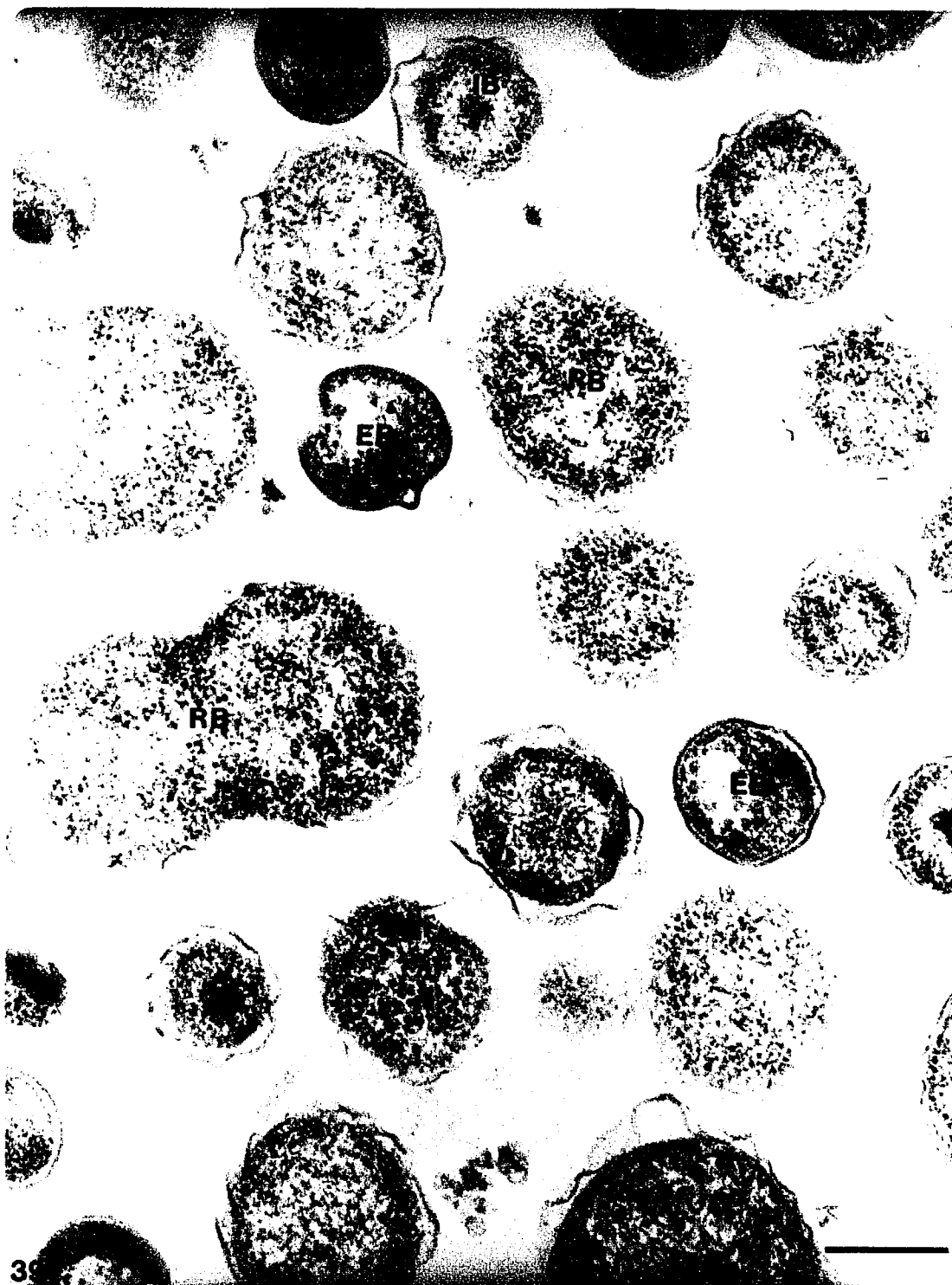


Fig. 40--Chorionic trophoblast (CT) from a placentomal hematoma of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined 18 days post-inoculation. A large chlamydial inclusion that contains numerous reticulate bodies and intermediate bodies with fewer elementary bodies occupies most of cytoplasm. Note that there is minimal cellular degeneration. Magnification = 4540 x.



Fig. 41--Chorionic trophoblast (CT) from a placentome of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined 18 days post-inoculation. A large membrane-bound chlamydial inclusion that contains numerous elementary and intermediate bodies (consistent with later stages of developmental cycle) occupies most of cytoplasm. Note advanced cellular degeneration characterized by swelling, vesiculation, loss of organelles, and displacement of the nucleus. Magnification = 4200 x.

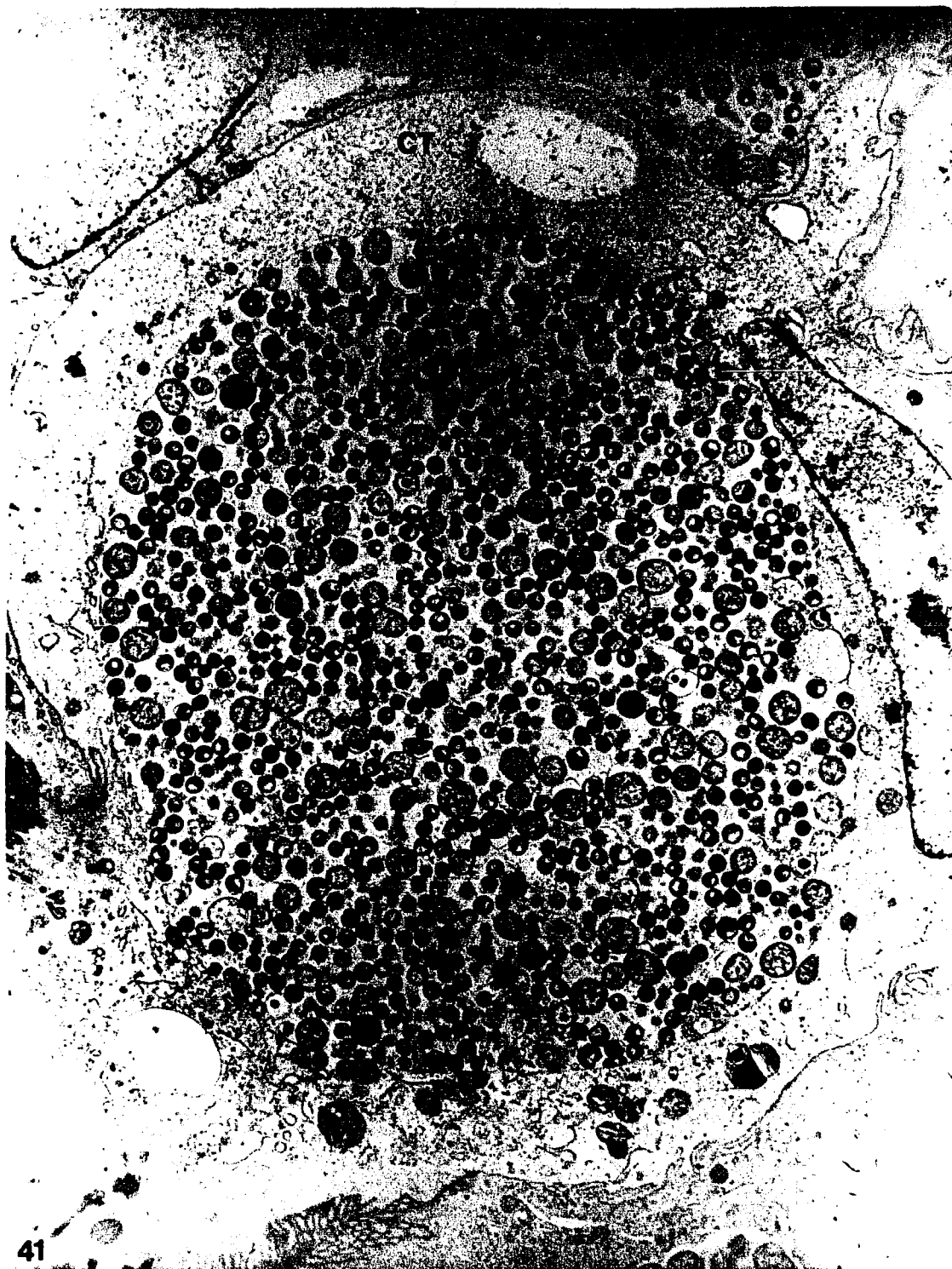
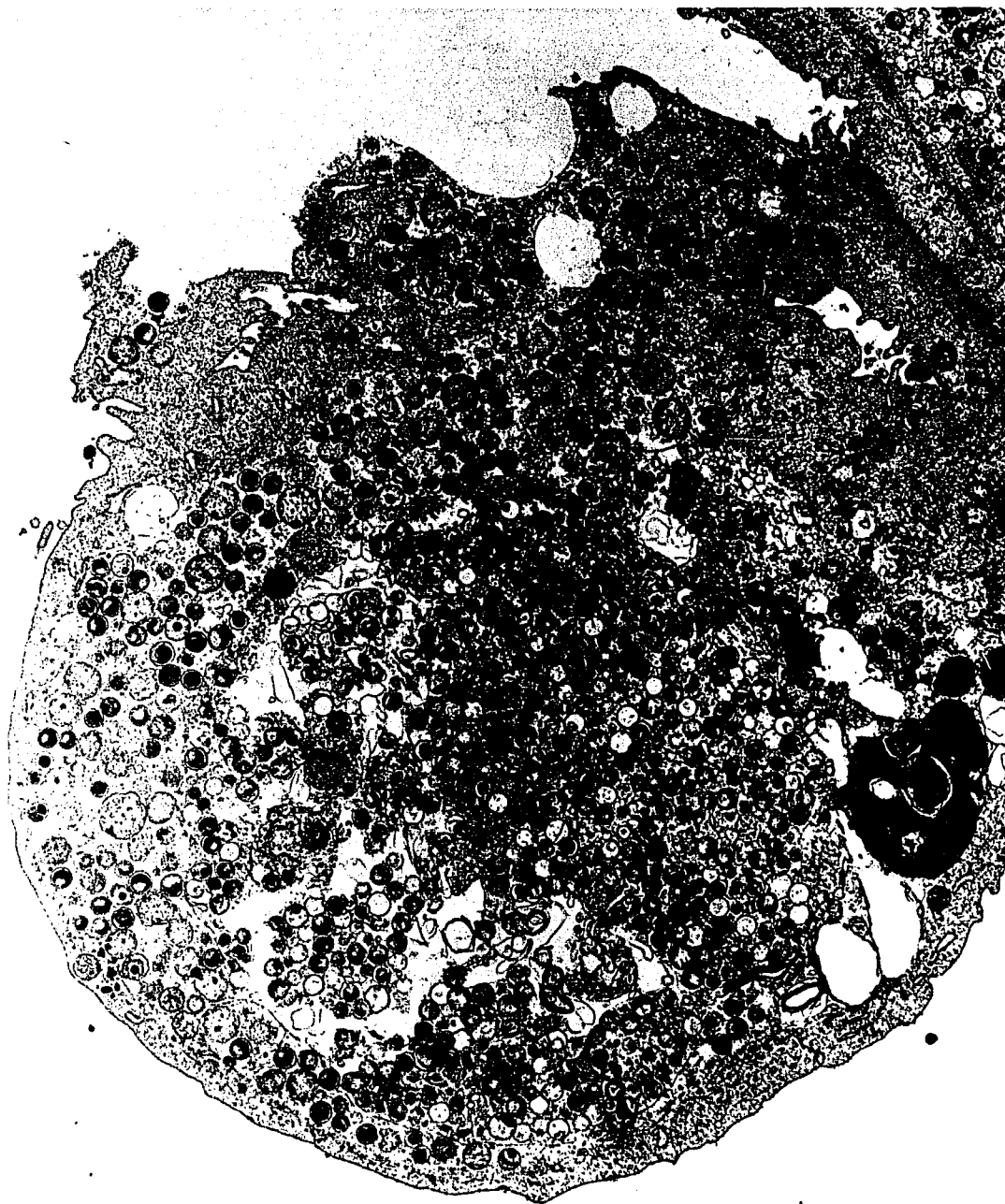


Fig. 42--Chorionic trophoblast from a placentomal hematoma of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined 24 days post-inoculation. A degenerate placentomal chorionic trophoblast (CT) is sloughing into the placentomal hematoma (HE). Note that inclusion membrane has ruptured and chlamydiae are present throughout the cytoplasm. Other degenerative changes include cell swelling, vesiculation, and loss of organelles and microvilli. Accumulation of membranous debris (arrows) in the cytoplasm were consistent with erythrophagocytic trophoblasts. Magnification = 5800 x.



HE

Fig. 43--Placentome from a ewe inoculated intravenously with 10^6 *C. psittaci* and examined 14 days post-inoculation. Erythrophagocytic chorionic trophoblasts (CT) lining placentomal hematomas contain membrane bound chlamydial inclusions that occupy much of the cytoplasm. Some trophoblasts contain crystalline inclusions and accumulations of membranous debris (MD) (consistent with erythrophagocytic chorionic trophoblasts). There are no abnormalities in the subepithelial mesenchymal tissues (stroma) (SM) or fetal capillaries (FC). Magnification = 2760 x.

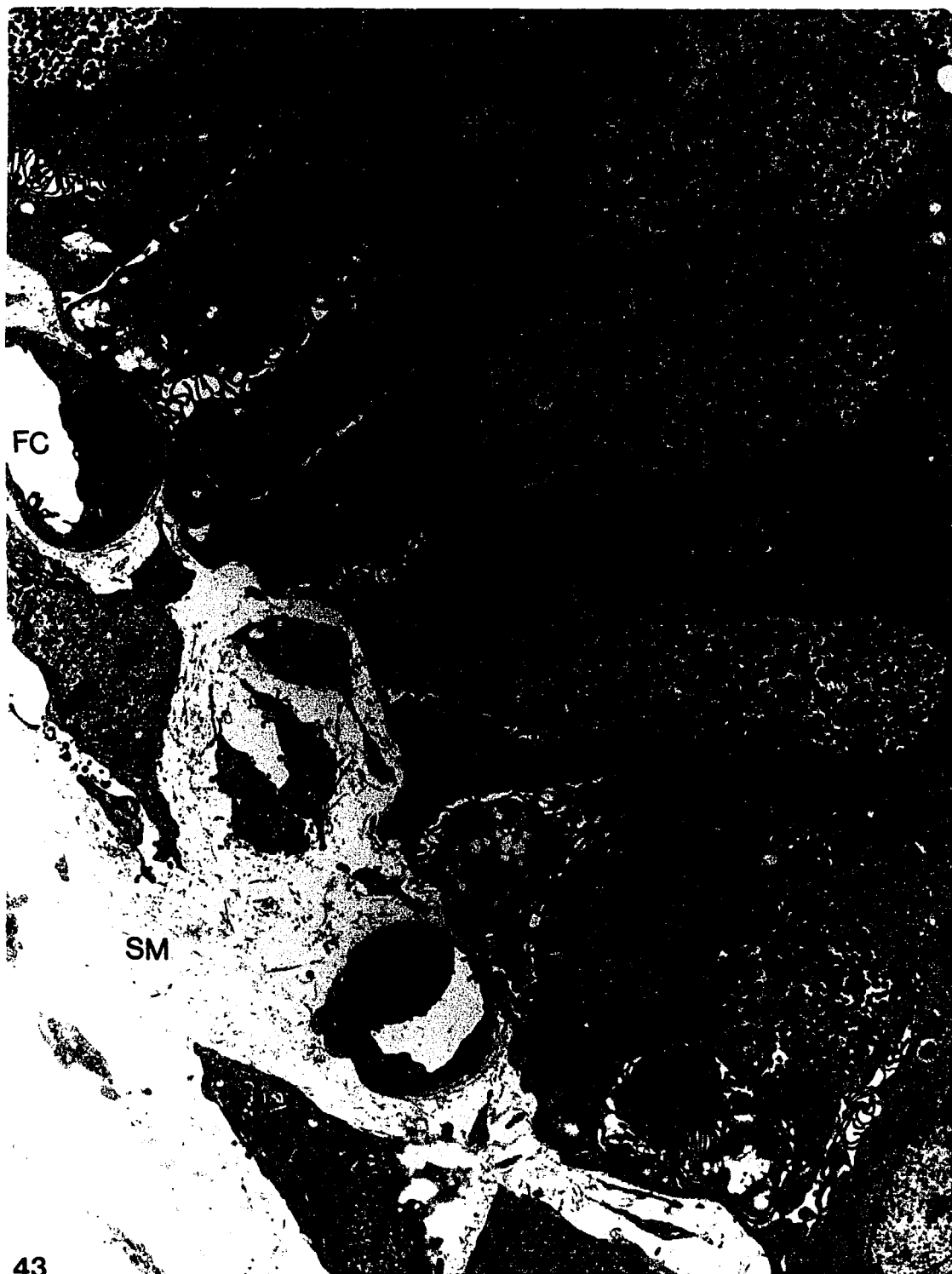


Fig. 44--Erythrophagocytic chorionic trophoblasts (CT) from a placentomal hematoma of a ewe inoculated intravenously with 10^6 *C. psittaci* and examined 14 days post-inoculation. Note membrane-bound chlamydial inclusions in perinuclear cytoplasm (early stages of developmental cycle-inclusions contain numerous reticulate bodies), membranous debris characteristic of remnants of phagocytized maternal erythrocytes (arrows) and minimal cellular degeneration. Magnification = 4540 x.

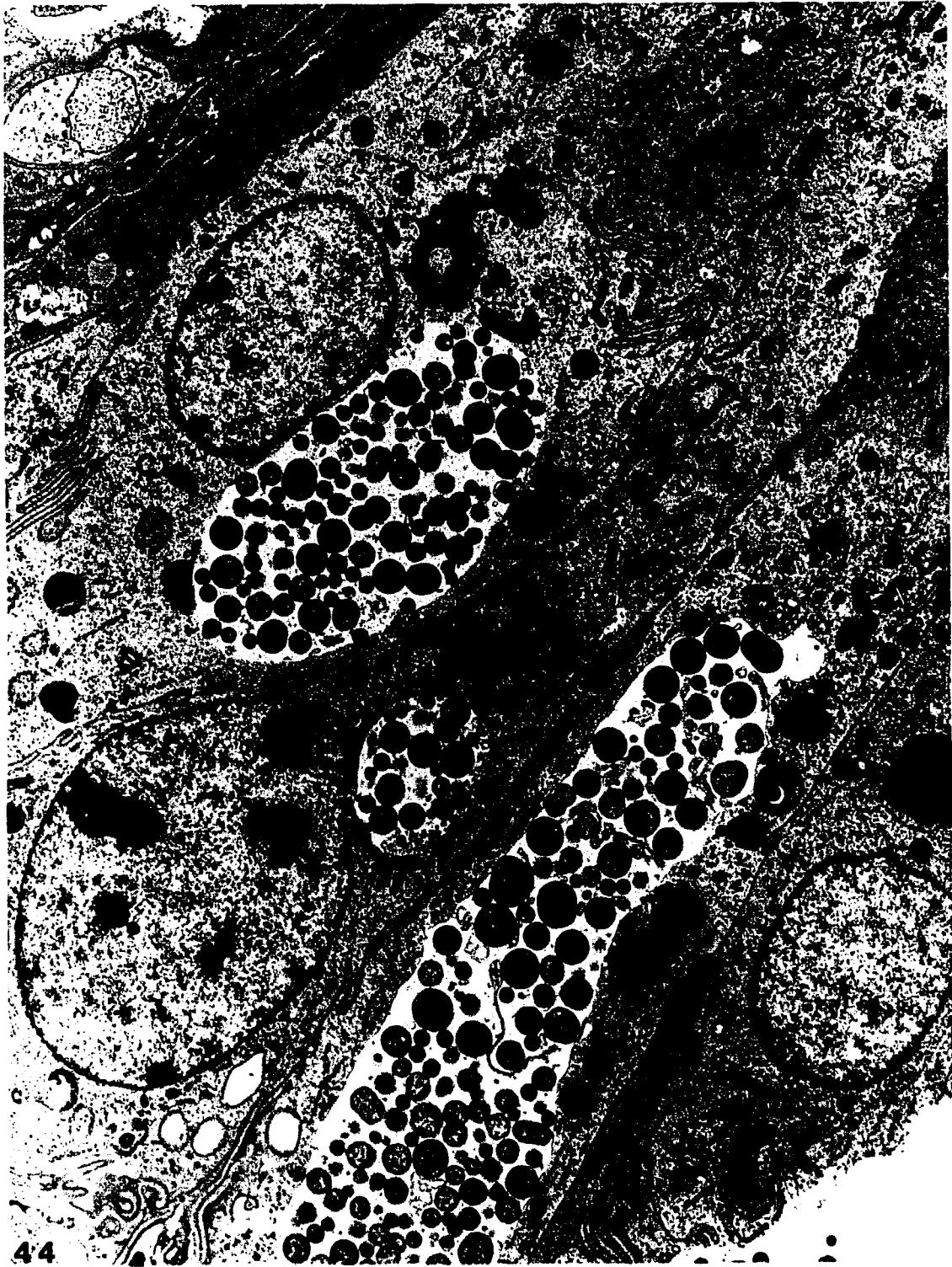


Fig. 45--Placentome of a ewe inoculated with intravenously with 10^6 *C. psittaci* and examined 18 days post-inoculation. Several chorionic trophoblasts (CT) lining placentomal hematoma contain membrane-bound chlamydial inclusions that occupy most of the cytoplasm and have displaced organelles to the periphery. Note that infected cells have fewer microvilli and there is cytoplasmic vacuolation. Placentomal hematoma (HE) contains free chlamydiae (arrows) and cell debris. Magnification = 4540 x.



Fig. 46--Placentome from Fig. 45. Subepithelial mesenchymal tissues (stroma) (SM) are edematous and contains increased numbers of fetal phagocytes (FP). Basement membrane is folded and chorionic trophoblasts (CT) contain chlamydial inclusions.

Magnification = 2760 x.



Fig. 47--Placentome from Fig. 45. Hematoma (HE) contains sloughed chlamydiae-infected degenerate chorionic trophoblasts (DT), cell debris, and free chlamydiae (arrows). Chorionic trophoblasts (CT) contain chlamydial inclusions. Magnification = 2760 x.



Fig. 48--Placentome of a ewe inoculated intravenously with 10^6 with *C. psittaci* and examined 28 days post-inoculation. The chorion is ulcerated and thick basement membrane (BM) is folded. Subepithelial mesenchymal tissues (stroma) (SM) are edematous and infiltrated with neutrophils. Hematoma (HE) contains cell debris, sloughed degenerate chlamydiae-infected trophoblasts (DT), and free chlamydiae (arrows).
Magnification = 2760 x.

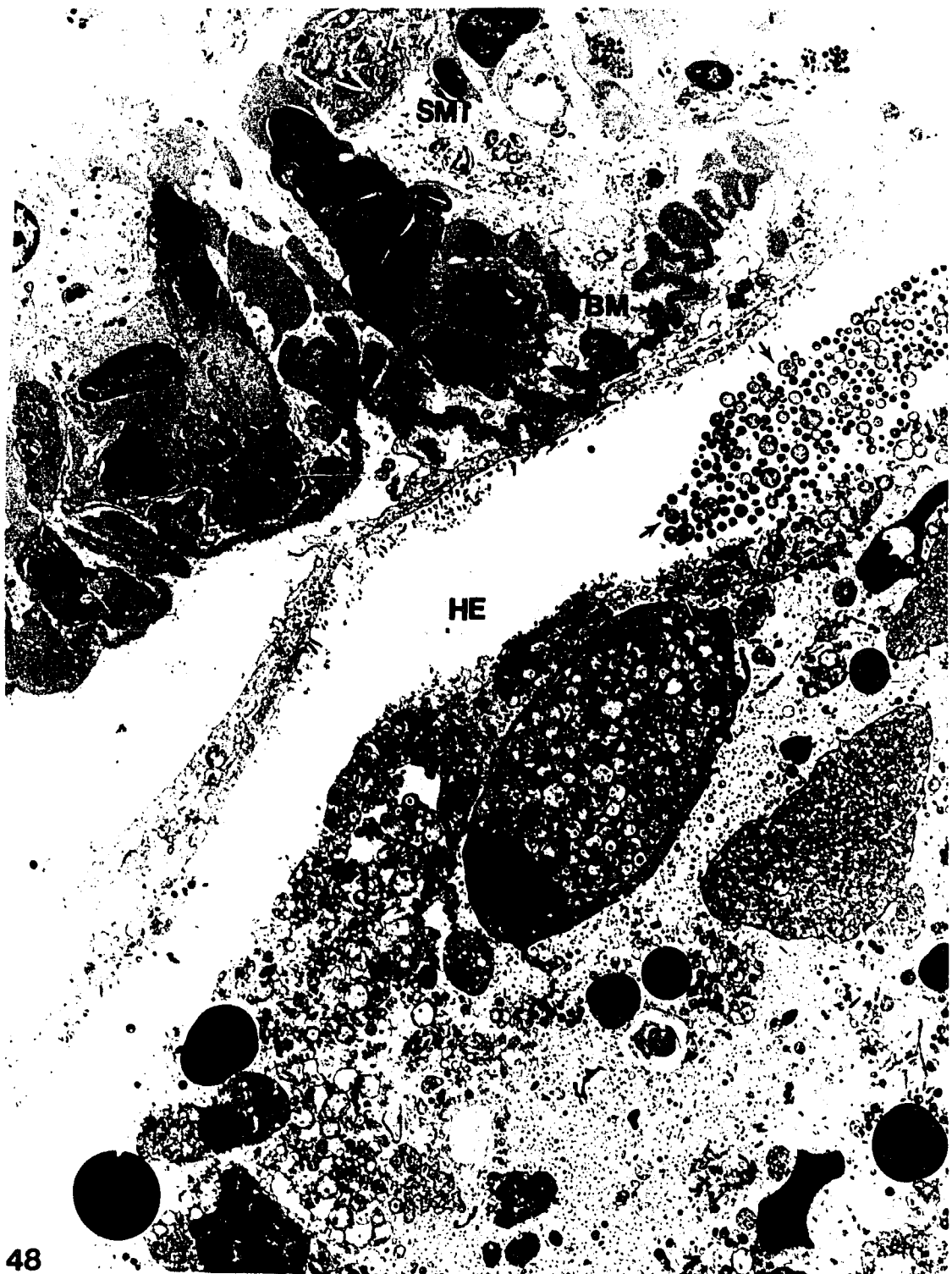


Fig. 49--Placentome of a ewe inoculated intravenously with 10^6 with *C. psittaci* and examined 43 days post-inoculation. Chorion of placentomal hematoma is ulcerated. Note that the thick basement membrane (BM arrows) is intact. Hematoma (HE) contains cell debris, cytoplasmic fragments of chlamydiae-infected trophoblasts (DT), and leukocytes (L). Subepithelial mesenchymal tissues (stroma)(SM) contains numerous degenerate leukocytes and cell debris. Magnification = 2760 x.

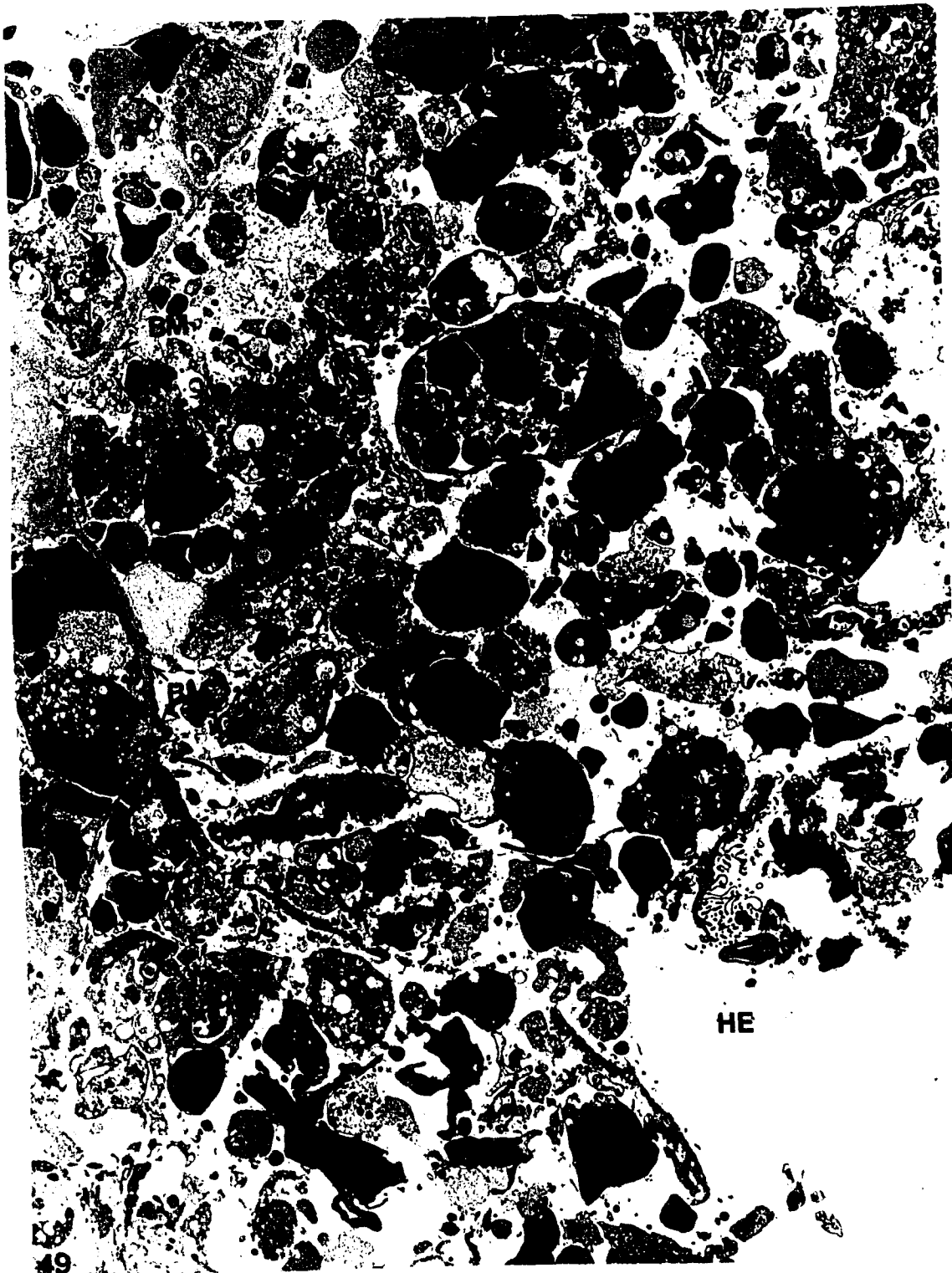


Fig. 50--Placentome from Fig. 49. Placentomal mesenchymal tissues (stroma) (SM) are edematous and contains numerous inflammatory cells, predominantly neutrophils.

Magnification = 2760 x.

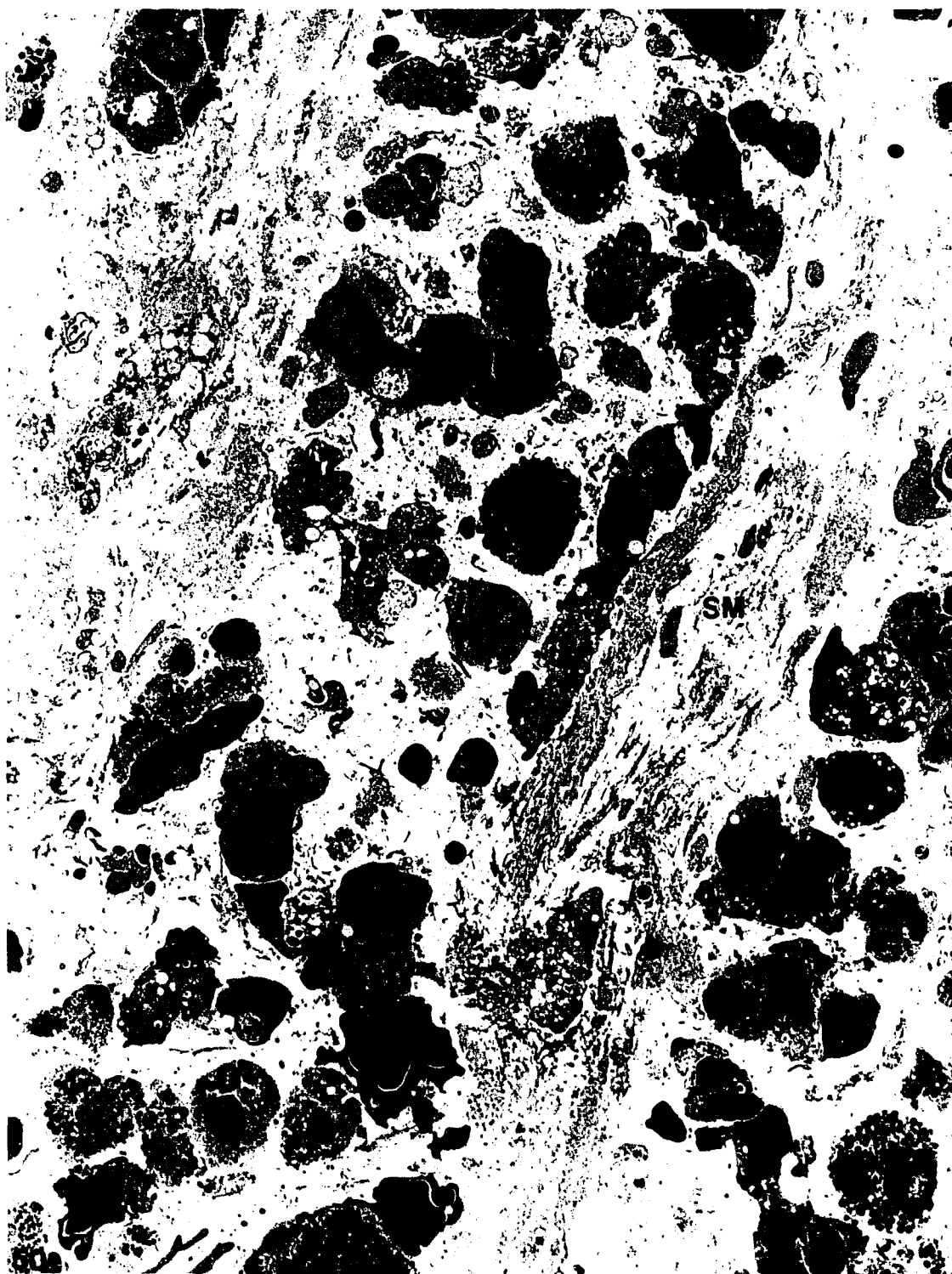


Fig. 51--Chorioallantoic membrane from the periplacentome of a ewe inoculated intravenously with *C. psittaci* and examined 21 days post-inoculation. Note the small membrane bound chlamydial inclusions in the cytoplasm of chorionic trophoblasts(CT). At this early stage of chlamydial development, there is minimal cellular degeneration. There are no abnormalities in fetal capillaries (FC), subepithelial mesenchymal tissues (stroma) (SM), and uterine space (lumen) (US). Magnification = 2760 x.

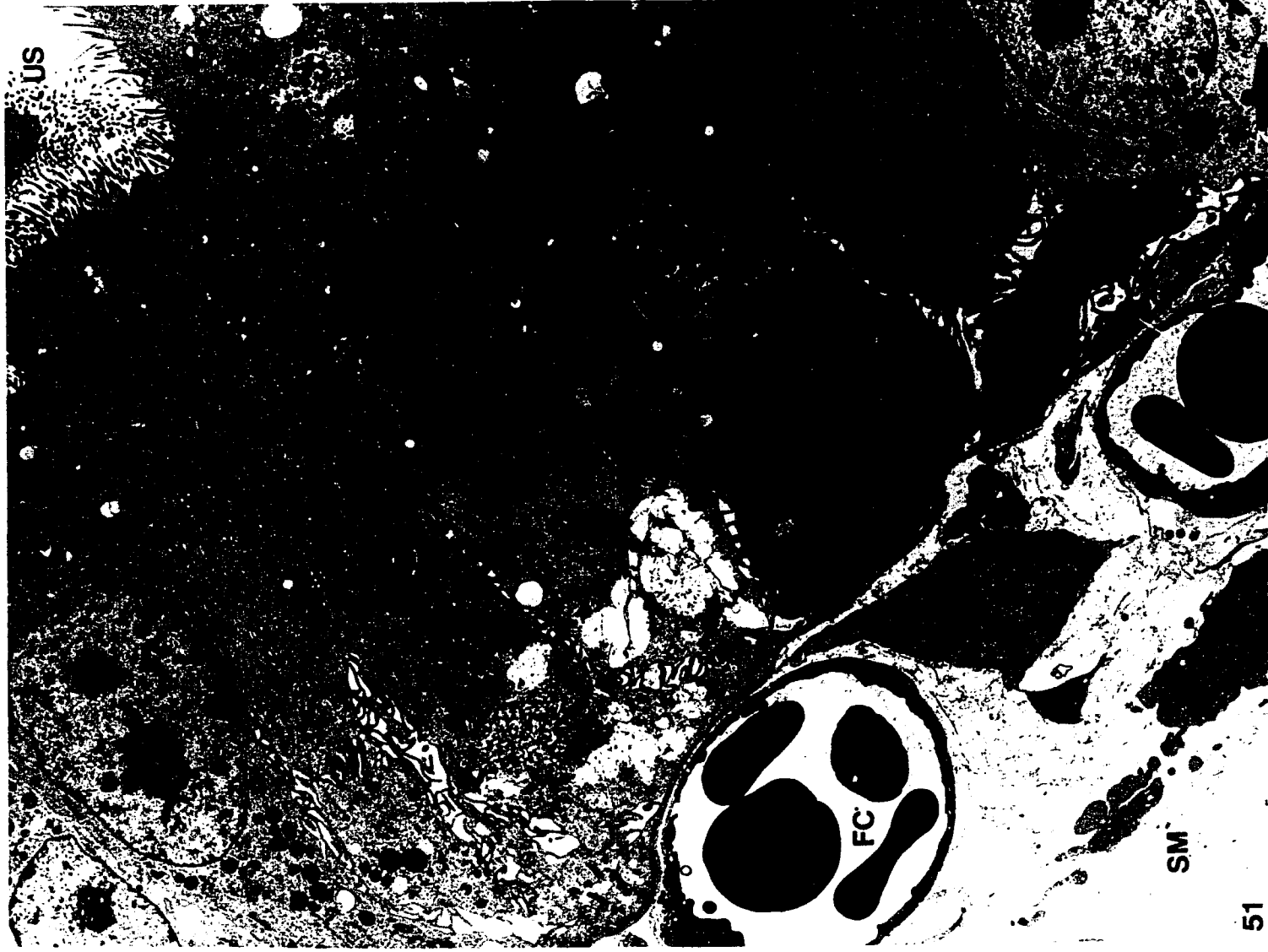


Fig. 52--Chorioallantoic membrane from the interplacentome of a ewe inoculated intravenously with *C. psittaci* and examined 43 days post-inoculation. Note that chorion is ulcerated and the basement membrane (BM arrows) is folded and irregular (arrows). A subepithelial fetal capillary is dilated and its endothelium is necrotic. Placental mesenchymal tissues (stroma) are edematous and contain neutrophils (N) and cell debris (probably from degenerate leukocytes). The uterine space (lumen) (US) contains cell debris. Magnification = 2760 x.

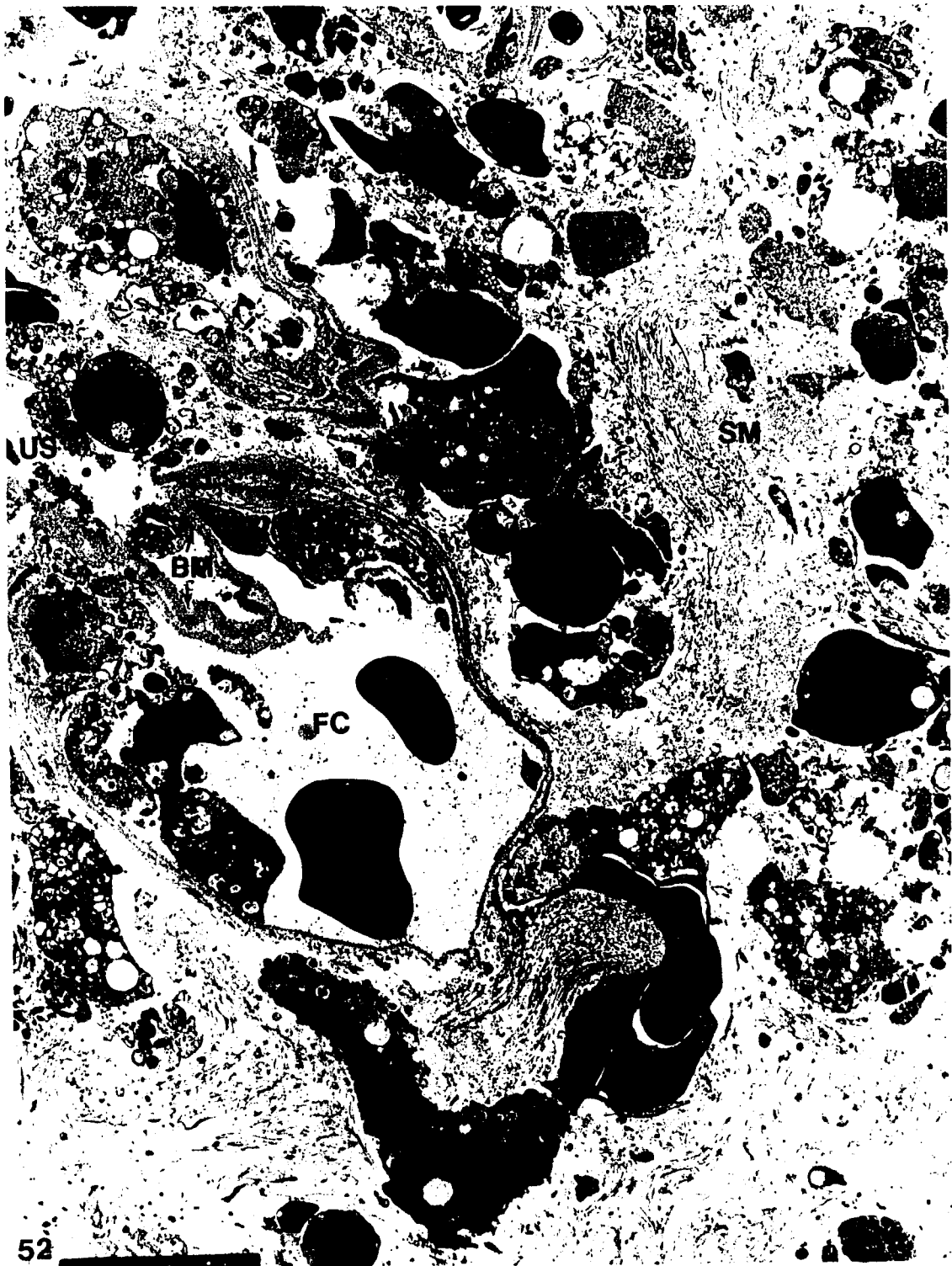
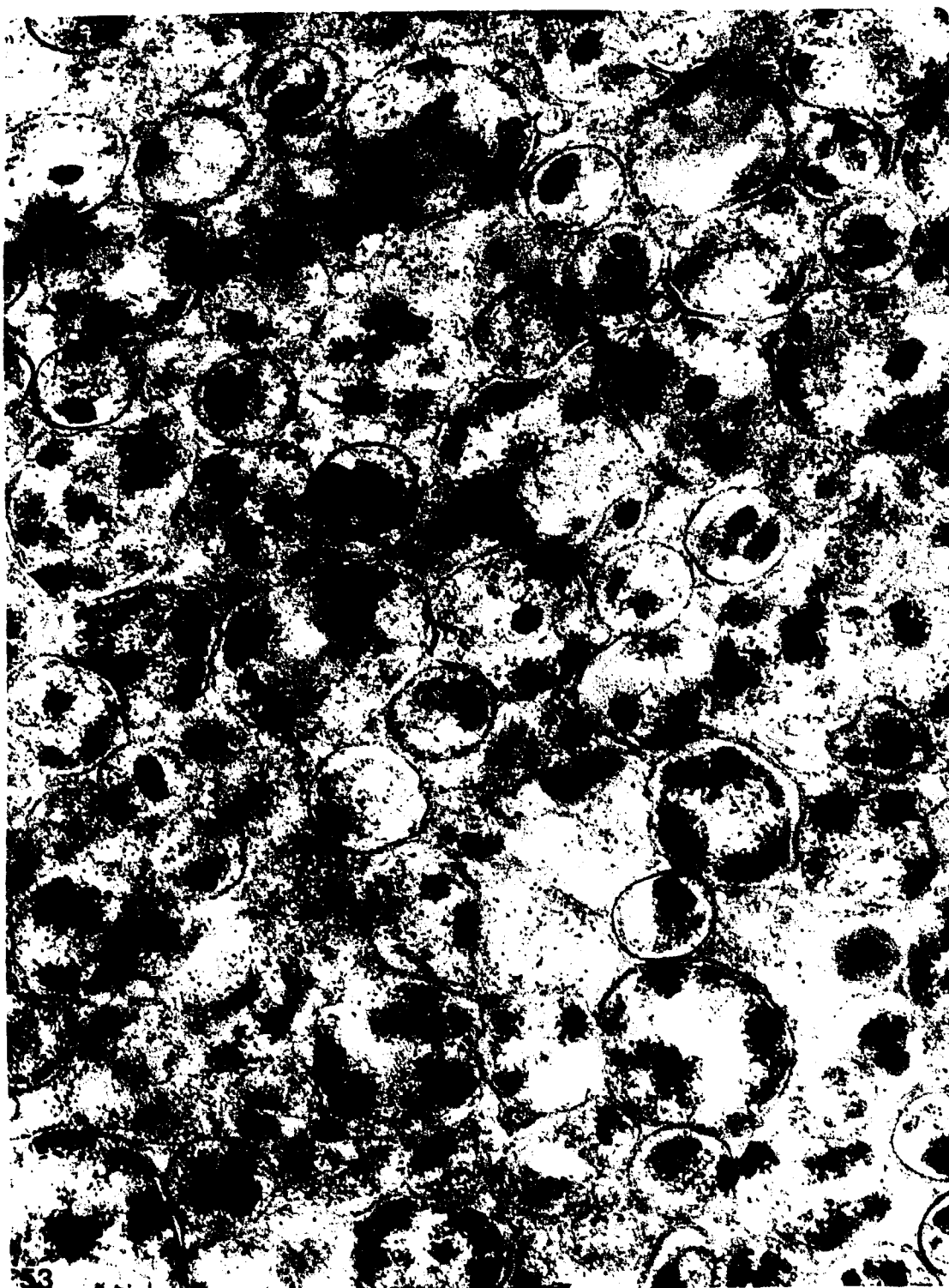


Fig. 53--Immunogold staining of chlamydiae in a placentomal hematoma of a ewe inoculated intravenously with 10^6 C. psittaci and examined 43 days post-inoculation. Immunogold particles attached to outer membranes and less often the cytoplasm of all chlamydial forms. Murine anti-chlamydial monoclonal antibodies. Magnification = 24500 x.



Discussion

The results of this study confirmed that *Chlamydia psittaci* first enters and replicates in chorionic trophoblasts lining placental hematomas. Within the hematoma, erythrophagocytic trophoblasts were initially infected. The infection quickly spread to trophoblasts of chorionic villi and the peri- and interplacentomes.

Placental hematomas provide an accessible route for chlamydiae from the maternal circulation to make direct contact with the chorionic epithelium.^{16,47,268,347} Well-developed hematomas are unique features of the placentomes of small domestic ruminants.^{6,17,19,32} Hematomas develop around the bases of chorionic villi and are composed of extravasated maternal blood that escapes from capillaries and larger blood vessels within tips of maternal septa. Erythrophagocytic chorionic trophoblasts lining hematomas are engaged in uptake of maternal blood from the 86th day of gestation until parturition.³⁹⁰ Chlamydiae probably are passively phagocytized by trophoblasts along with maternal erythrocytes. However, active entry (uptake) of chlamydiae cannot be rule-out. Other ultrastructural studies have demonstrated active uptake of *C. psittaci* into intestinal epithelial cells of newborn calves.⁹⁶⁻⁹⁹

Chorionic trophoblasts provided a suitable environment for replication of chlamydiae. Chlamydiae clearly replicated within membrane-bound endosomes in the cytoplasm of chorionic trophoblasts. Early stages of infection were not associated with degenerative changes in the host cell. However, in later stages of the developmental cycle there was progressive cellular degeneration characterized by swelling, displacement and loss of organelles, dilation and vesiculation of endoplasmic reticulum, loss of microvilli, and

nuclear swelling or pyknosis. The host cell inevitably was destroyed by massive chlamydial development. Chlamydiae were released by rupture and lysis of cytoplasmic inclusion membranes. Todd and Storz demonstrated free lysosomal enzymes in the host cell cytoplasm at the time of release of chlamydiae.³⁷¹ However, it is not clear whether this enzyme release is a cause or a consequence of chlamydial release.

In this study, chlamydial infection was limited to chorionic trophoblasts. This was in marked contrast to the invasiveness described by Doughri and Storz in their study of infection of neonatal bovine gut with *C. psittaci*.⁹⁷⁻⁹⁹ They found chlamydiae not only within mucosal epithelial cells of the gut, but also within cells in the lamina propria (i.e., macrophages, plasma cells, fibroblasts, neutrophils), and lymphatic endothelium.^{97,98} This discrepancy emphasizes the marked differences in the infectious process and/or tissue tropism that may exist between various strains of *C. psittaci*.

Once chlamydial infection was established in the placentomes, infected chorionic trophoblasts ruptured or sloughed releasing large numbers of chlamydiae into placentomal hematomas. No doubt, accumulations of exudate containing large numbers of chlamydiae facilitated the spread of the infection within the placentome and to the peri- and interplacentome. Placentomal hematomas are continuous with uterine spaces (lumen) of the periplacentomes. Adjacent chorionic trophoblasts probably were infected by endocytosis of free chlamydiae and/or fragments of chlamydiae-infected trophoblasts from the hematomas and uterine spaces. Spread of the chlamydial infection in the interplacentome probably is enhanced separation of the endometrium and chorioallantoic epithelium associated with

inflammation. Although in this study no chlamydiae were identified in blood vessels, hematogenous spread of chlamydiae to other areas of the placenta cannot be ruled-out. The close proximity of placental capillaries to infected and /or ulcerated chorion allowed ready access of chlamydiae to the fetal circulation. A study of enteric infection of *C. psittaci* in calves, demonstrated that *C. psittaci* infected lymphatic endothelial cells of the lymphatic system and liberated chlamydiae into the lymphatic circulation leading to chlamydemia and systemic infection.^{97,98}

Chlamydial infection of the placenta resulted in an intense neutrophilic inflammatory response. Minimal inflammation was associated with early infections, but as trophoblasts were lost and/or basement membranes were damaged, an intense inflammatory response was initiated. Neutrophilic exudation probably occurred in response to dead or degenerating chlamydiae-infected trophoblasts. Chlamydiae can activate complement to generate C5a, a potent chemotactant for neutrophils.²⁴² This mechanism may be partially responsible for the intense neutrophilic inflammatory response associated with acute chlamydial infections. Additionally, toxins released from large numbers of chlamydiae may have contributed to the inflammatory changes in these placentas including influx of neutrophils, edema, vascular degeneration may be due to toxins released from large numbers of chlamydiae. The lipopolysaccharide(LPS) of chlamydiae shares many biologic properties with endotoxin of other gram-negative bacteria, including complement activation, mouse lethality, and abortifacient properties.^{41,57,269,270}

The large numbers of chlamydiae in infected placentas may have been due the failure of

leukocytes to destroy them and limit their replication. Surprisingly, no phagocytized chlamydiae were identified in inflammatory cells. This was in contrast to ultrastructural studies of enteric chlamydial infections in calves in which chlamydiae were present in macrophages, plasma cells, and neutrophils.^{97,98} Characteristics of the chlamydial surface may be responsible for the minimal interaction between chlamydiae and ovine fetal neutrophils. Other studies have demonstrated that placental leukocytes are unable to arrest multiplication of many facultative intracellular pathogens such as *Brucella*, *Listeria*, and *Salmonella*.^{73,221} Additionally, fetal leukocytes may be immature and therefore, not fully functional. Studies have shown that ovine fetal neutrophil enzyme systems are not present until 135 days of gestational age and monocyte enzyme systems until after birth.³¹⁰ Additionally, *in vitro* studies have suggested that serum containing IgG > 100 mg/dl is needed for phagocytosis of *C. trachomatis* by human neutrophils.¹⁵¹ Perhaps low levels of IgG in the ovine placenta also may limit neutrophil function. The role of cell-mediated immunity in placental resistance to chlamydial infection is uncertain.

In summary, the evidence of this study indicates that strain OSP of *C. psittaci* replicates in chorionic trophoblasts. Erythrophagocytic trophoblasts of placentomal hematomas were initially infected. Intracellular proliferation of chlamydiae resulted in progressive degeneration of trophoblasts and the infection quickly spread to chorionic trophoblasts of the periplacentome and interplacentome. Neutrophilic inflammation was the predominant inflammatory response, but seemingly was ineffective at limiting chlamydial replication.

**6. EVALUATION OF AN ENZYME IMMUNOASSAY FOR
DETECTION OF *CHLAMYDIA PSITTACI* IN VAGINAL SECRETIONS,
PLACENTAS, AND FETAL TISSUES FROM ABORTING EWES**

Summary

A commercially available enzyme immunoassay (EIA) for the detection of *Chlamydia trachomatis* in human urogenital and conjunctival specimens was compared with isolation in cell culture for the detection of *Chlamydia psittaci* in vaginal and placental swabs from aborting ewes and swabs of aborted fetal tissues. The EIA on vaginal swabs collected from 10 ewes experimentally infected with *C. psittaci* had a sensitivity of 85.7% and a specificity of 85.7% when compared to isolation of chlamydiae in cell culture. Vaginal swabs collected at the time of abortion or within 3 days were the best samples for detection of chlamydial infection. Twenty-nine vaginal swabs collected during this period were strongly EIA-positive, and chlamydiae were isolated from 28. The EIA on vaginal swabs from 78 field cases of abortion had a sensitivity of 78.0% and a specificity of 76.8%. The EIA on swabs of cotyledons from 65 placentas had a sensitivity of 100% and a specificity of 75.0% compared with isolation in cell culture. Swabs of fetal tissues or body fluids from 10 aborted fetuses or weak lambs were evaluated with the EIA; it had a sensitivity of 26.6% and a specificity of 88.1% compared with isolation in cell culture. Limitations of the EIA are discussed.

Introduction

Chlamydial abortion, known as enzootic abortion of ewes, is a major cause of fetal loss in sheep.¹⁻⁷ This disease occurs worldwide and in many areas is the most commonly diagnosed cause of ovine abortion.^{1,8} Enzootic abortion of ewes is caused by ovine strains of *C. psittaci* that have a predilection for placental tissues.^{1,4,6} Placental and fetal infections with this gram-negative bacteria result in abortion or the birth of stillborn, moribund, or weak lambs.^{5,9-11} In severe outbreaks, up to one-third of the ewes may be affected. Endemic disease in a flock may result in abortion rates of 1-5%.

Diagnosis of chlamydial abortion is often based upon characteristic gross and light microscopic lesions in the placenta and demonstration of organisms in direct placental smears or in histologic sections.^{3,11} Other useful techniques include serology and examination of fluorescent antibody-stained impression smears or frozen sections of fetal tissues or placenta. Fetal lesions often are nonspecific. A diagnosis of chlamydial abortion is confirmed by isolation of the organism in embryonated eggs or cell culture. These isolation procedures are time consuming and costly. The placenta is the tissue of choice for chlamydial isolation; fetal tissues often contain low numbers of viable organisms.

Chlamydiae are readily inactivated in the course of intrauterine events that lead to fetal death.^{2,7} In addition, bacterial contamination and unfavorable transport conditions further reduce the possibility of isolating this organism.¹²

There is need for rapid and reliable techniques for direct (non-culture) detection of chlamydiae in diagnostic specimens from sheep. Several commercially available tests,

including microimmunofluorescence techniques for urethral and cervical smears,¹³⁻¹⁶ immunohistochemical procedures for detection of chlamydial inclusions in endometrial and cervical biopsies,¹⁷ enzyme immunoassays,^{13,18-25} and DNA probe assays,²⁶ have been developed for detection of *C. trachomatis* infection in humans. These tests have several advantages: speed of diagnosis, potential for automation, and ease of specimen transport.

Currently, diagnostic tests for direct detection of *C. psittaci* in animal specimens are not commercially available. Experimentally, an enzyme-linked immunoabsorbent assay that utilizes monoclonal antibodies for an antigen capture system has been developed for diagnosis of chlamydial abortions in sheep and goats.²⁷ Recently, another enzyme immunoassay for *C. trachomatis* has been used to detect *C. psittaci* in conjunctival swabs from cats.²⁸

In this study, an enzyme immunoassay (EIA) designed to detect the group antigen of *C. trachomatis* in human urethral, endocervical, and conjunctival swabs was compared with isolation in cell culture for detection of *C. psittaci* in ovine vaginal and placental swabs and in aborted fetal tissues.

Materials and Methods

Collection and Handling of Specimens

Vaginal swabs from ewes infected experimentally with C. psittaci: Ten mature crossbred ewes (9 pregnant [8 mid-gestation, 1 early gestation], 1 non-pregnant) were inoculated intravenously in the jugular veins with 1.0 ml of 0.01 M phosphate-buffered saline (PBS) (pH 7.4) containing 10⁶ inclusion forming units of strain B577 of *C. psittaci*.

Ewes were housed in isolation and observed twice daily for clinical signs of disease. All ewes were tested for chlamydial antibodies with a complement fixation procedure (National Veterinary Services Laboratories, Ames, IA) prior to infection and results were negative.

After inoculation, duplicate vaginal swabs were collected 2-3 times per week until abortion or lambing, daily for 3-5 days after abortion, and 2-4 weeks until euthanasia and necropsy. One swab was immediately placed in a 12 x 75-mm polystyrene round-bottom tube containing 1 ml of Bovarnick's phosphate buffer (pH 7.4) and held at 4°C for up to 24 hours before isolation was attempted. The other swab was placed in a specimen tube (supplied with the EIA kit) and stored at 4° C for up to 5 days. These swabs were tested for chlamydial antigen with the EIA, and the results compared with isolation in cell culture.

Vaginal swabs from non-infected ewes lambing normally: Two vaginal swabs were collected simultaneously from each of 16 clinically normal ewes within 4 hours after lambing. One swab was used for chlamydial isolation in cell culture and the other was tested by EIA. There was no clinical evidence of chlamydial abortions in the flock history.

Vaginal swabs from field cases of abortion: Two vaginal swabs from each of 78 aborted ewes were collected by veterinary practitioners. Swabs for chlamydial isolation in cell culture were placed in 12 x 75-mm polystyrene tubes containing 1-2 ml of Bovarnick's phosphate buffer. Specimens for EIA were placed in collection tubes and all samples were returned by mail and stored at 4°C before testing.

Placental swabs from experimentally infected ewes and field cases of abortion:

Swabs of placental cotyledons were collected from experimentally infected ewes and tested for chlamydial antigen with the EIA. These cotyledons also were used for chlamydial isolation in cell culture. Swabs for EIA analysis were also collected from ovine placentas that were submitted to the Iowa Veterinary Diagnostic Laboratory, Ames, Iowa.

Representative samples were collected and submitted to the National Veterinary Services Laboratories, Ames, Iowa, for chlamydial isolation in cell culture.

Swabs of fetal tissues and body fluids: Swabs of abomasal contents, lungs, liver, abdominal or thoracic fluids, and conjunctiva were collected from aborted fetuses and weak lambs from experimentally infected ewes. They were stored at 4°C for up to 5 days and tested for chlamydial antigen by EIA. Representative samples of tissues/fluids were used for chlamydial isolation in cell culture.

Enzyme Immunoassay (EIA)^a

The EIA (Chlamydiazyme®, Abbott Laboratories, North Chicago, IL) was performed according to the manufacturer's instructions. Briefly, 1 ml of dilution buffer (provided in the EIA kit) was added to each tube containing a swab specimen. Aliquots (200 µl) of vortexed specimens and controls (3 negatives and 1 positive-supplied with the EIA kit) were transferred into 1 well of a plastic microtiter plate. This test uses treated polystyrene beads that bind chlamydial antigen. The nature of the coating of these beads was not specified by the manufacturer. One bead was added to each well and the plate was incubated in a 37°C water bath for 60 minutes. Each bead was washed and 200 µl of rabbit anti-chlamydiae

antiserum was added to each well. After incubation for 60 minutes in a 37° C water bath, beads were washed and reacted with 200 µl of horseradish peroxidase-conjugated antibody to rabbit IgG. After a third incubation of 60 minutes in a 37°C water bath and washing, the beads were transferred to individual clean plastic tubes (supplied with EIA kit) and 300 µl of peroxidase substrate was added to each. The reaction was stopped after incubation for 30 minutes at room temperature (25° C). Results were determined using a spectrophotometer (Quantum Spectrophotometer®, Abbott Laboratories, North Chicago, IL) at 492 nm. A specimen was considered positive if the optical density (OD) exceeded the mean of 3 negative control determinations plus 0.10. Positive reactions were graded as follows: slight, OD 0.20-0.40; moderate, OD 0.40-1.0; and strong, OD 1.0-2.0.

Chlamydial Isolation

Vaginal swabs: Vaginal swabs were placed in 2 ml of cell culture medium composed of Eagle's minimum essential medium with Earle's balanced salts containing 20 mmol HEPES(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid); 5% fetal bovine serum; 5.4 mg/liter of glucose; 292 mg/liter of glutamine; 2 µl of amphotericin B; 500 µg/ml each of streptomycin, vancomycin, and kanamycin; and 0.5 µg of cyclohexamide. They were refrigerated overnight at 4° C. After vigorous vortex mixing the swabs were expressed and discarded. The fluid was centrifuged for 10 minutes at 700 x g at 20°C. The middle 1 ml of the supernatant was removed and 200 µl was inoculated into each of 2 wells of a 96-well plate containing either 24-hr confluent African green monkey (Vero) or mouse fibroblast (McCoy) cell monolayers. Inoculations were done in 4 plates to provide cultures for

examination at different times and/or for repassage. Inoculated plates were centrifuged at 1,000 x g for 60 minutes at 35°C. The inoculum was removed, fresh medium was added, and plates were incubated at 37°C in 4% CO₂. On days 3 and 6 one plate was fixed with a 50% acetone-50% methanol mixture for 5-10 minutes. Monolayers were stained and examined for chlamydial inclusions by an indirect fluorescent antibody (FA) technique using a group-reactive mouse monoclonal antibody to ovine chlamydial strain B577 and a fluorescein-conjugated anti-mouse IgG^c immunoglobulin (IgG-heavy and light chain specific) (Organon Teknika, Malvern, PA) at a dilution of 1:30. They were examined for inclusions with an epifluorescence microscope at magnifications 400 to 600 x. The number of chlamydial inclusions per well on initial isolation was used to estimate the level of tissue infection. Positive cultures were graded as follows: low <5 inclusions per well; moderate, 5-15 inclusions per well; and high, >15 inclusions per well.

Placental and fetal tissues: Approximately 1 gram of each tissue was minced using a mortar and pestle. A 10% (weight/volume) suspension was prepared in cell culture medium. After overnight refrigeration at 4° C, samples were processed as previously described.

Placental Impression Smears

Exudate from placental cotyledonary surfaces were stained by the method of Gimenez and examined for chlamydial organisms and examined for chlamydial organisms by light microscopy.³¹

Results

Vaginal Swabs

Vaginal swabs from ewes infected experimentally with C. psittaci: Eight of nine infected pregnant ewes either aborted in late gestation or gave birth to small (2.5-3.5 kg) weak lambs. Macroscopic placental changes included multifocal necrosis and thickening of cotyledons and intercotyledonary placentas, edema, hyperemia, and hemorrhage. Variable amounts of a thick yellow-brown exudate was present on the chorionic surface. Gimenez-stained smears of this exudate demonstrated numerous 0.3-0.6 μm coccoid organisms that were morphologically compatible with chlamydiae. Additionally, high numbers of chlamydiae were isolated from these placentas. One pregnant ewe was euthanized and necropsied at 65 days post-inoculation. It contained 2 normal fetuses that were approximately 10-12 cm in length. There were no gross or microscopic lesions in their placentas, however, low numbers of chlamydiae were isolated from the amniotic fluid.

Results from 252 vaginal swabs collected from 10 experimentally infected ewes are shown in Table 8. These samples represented swabs collected up to 90 days before and 60 days after abortion. The EIA had a sensitivity of 85.7% and a specificity of 85.7% compared with isolation in cell culture.

Generally, vaginal swabs collected from infected ewes more than 3 days before abortion or lambing were EIA and isolation negative. However, 9 of the 118 swabs collected during this period were slightly to moderately positive (OD 0.2-0.9); low numbers of chlamydiae were isolated from 5 of these swabs. All swabs (15) collected from the infected non-

pregnant ewe were EIA- and isolation negative. Vaginal swabs from 4 ewes (Nos. 3,6,12, and 14) were strongly EIA-positive ($OD > 2.0$) and culture positive 1-3 days prior to abortion or lambing (Fig. 54). These strongly positive vaginal samples coincided with the appearance of the yellow-brown vaginal discharges.

Vaginal swabs (29) collected from the 8 experimentally infected ewes at abortion and up to 3 days afterward (post abortion days = PAD) were strongly EIA-positive (OD 1.8- >2.0) (Fig. 54). Vaginal swabs collected from 3 ewes (Nos. 1,3, and 5) were EIA and isolation positive for up to 14 PAD. Swab specimens from ewe no. 15 remained strongly EIA-positive ($OD > 2$) until necropsy on PAD 19 (Fig. 54); however, chlamydiae were not isolated after PAD 9. Positive EIA results were obtained from samples from ewe no. 6 until PAD 34 and isolations were made to PAD 19. Overall, vaginal swabs collected from the 8 infected aborting ewes were EIA-positive for an average of 10 PAD (range = 3-34 days). Chlamydiae were isolated from vaginal swabs for an average of 8 PAD (range = 3-19 days).

Vaginal swabs from non-infected normal ewes: Vaginal swabs collected from 16 clinically normal ewes after lambing were EIA- and isolation-negative.

Vaginal swabs from other (field cases) aborting ewes: Seventy-eight vaginal swabs were examined by EIA and isolation in cell culture. Fifty-eight of these samples were collected from 5 to 21 PAD. The EIA had a sensitivity of 78.0% and a specificity of 76.8% compared with isolation in cell culture (Table 8).

Placental Swabs from Experimentally Infected ewes and Field Cases of Abortion

Swabs of cotyledons from 65 placentas (8 experimentally infected and 57 field cases) were examined by EIA and isolation in cell culture. The results are shown in Table 8. Placental swabs from experimentally infected ewes and 5 isolation-confirmed field cases of chlamydial abortion were strongly EIA-positive (OD>2.0). Swabs from 13 placentas were EIA-positive and isolation negative. A severe necrotizing and suppurative placentitis was present in 5 of these specimens. Gimenez-stained microscopic sections revealed numerous 0.3-0.6- μ m red intracellular and extracellular coccoid organisms consistent with chlamydiae. No significant gross or microscopic lesions were present in 8 EIA-positive placentas. The sensitivity and specificity of the EIA on placental swabs was 100% and 75.0%, respectively (Table 8).

Swabs of Tissues and Body Fluids from Ovine Fetuses Infected with C. psittaci

Fifty-seven tissues or body fluids from 10 fetuses or lambs were tested with the EIA and isolation in cell culture (Table 8). Low numbers of chlamydiae were isolated from a total of 15 samples from 6 fetuses. Chlamydiae were isolated from the liver and lung from 5 fetuses. Isolations were also made from abomasal contents and conjunctival swabs from 2 fetuses. One isolation was made from the kidney. Compared with isolation, the EIA had a sensitivity of 26.6% and a specificity of 88.1%.

Table 8. Comparison of results by enzyme immunoassay and isolation for detection of *C. psittaci*

Sample	Number of specimens				Total	Sensitivity	Specificity
	ISO ^a , EIA ^b -	ISO+, EIA+	ISO+, EIA-	ISO-, EIA+			
Vaginal swabs							
Experimental ^c	162	54	9	27	252	85.7%	85.7%
Field ^d	53	7	2	16	78	77.7%	76.8%
Combined	215	61	11	43	330	84.7%	83.3%
Placentas ^e	39	13	0	13	65	100.0%	75.0%
Fetal tissues ^f	37	4	11	5	57	26.6%	88.1%

^a ISO = isolation in cell culture; - = negative isolation; + = positive isolation.

^b EIA = enzyme immunoassay; - = negative result; + = positive result.

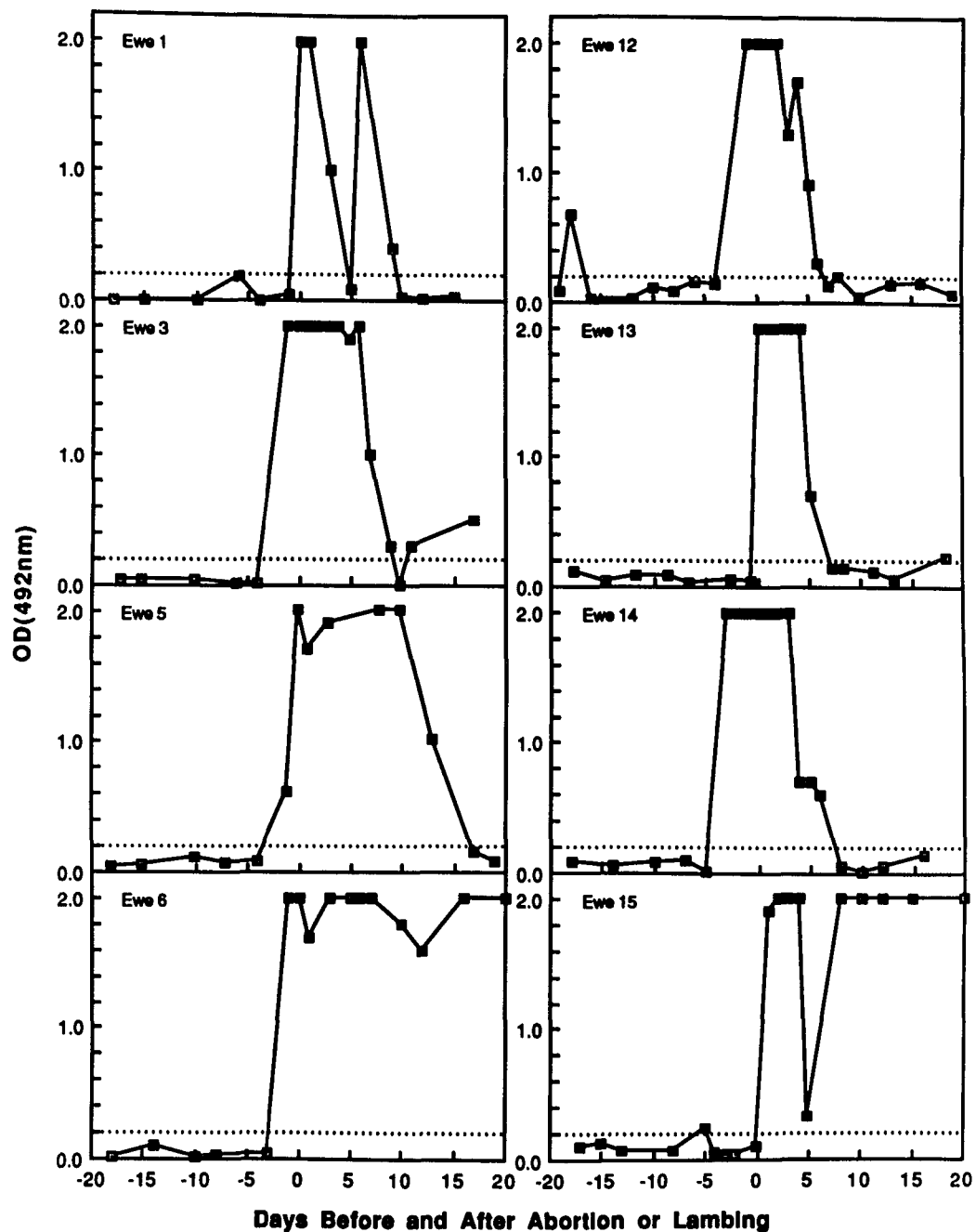
^c Swabs collected over a period of time from 10 experimentally infected ewes and specimens from 16 clinically normal ewes at lambing.

^d Samples collected from 78 field cases of abortion.

^e Swabs of placental cotyledons from 8 experimentally infected ewes and 57 field cases of abortion.

^f Ten fetuses from 8 experimentally inoculated ewes.

Fig 54.--EIA results on vaginal swabs collected from ewes experimentally inoculated with strain B577 *C. psittaci*. Day 0 = abortion or lambing, OD = optical density, OD < 0.2 = negative, OD 0.2-0.4 = slight positive, OD 0.4-1.0 = moderately positive, OD 1.0-2.0 = strongly positive.



Discussion

One problem when evaluating direct diagnostic tests for chlamydiae is the absence of a highly sensitive reference test. Isolation is the standard to which direct tests should be compared; however, under optimal conditions, it is estimated to have a sensitivity of only 70-80%.^{16,22} Contaminating bacteria, specific antibodies,^{24,29} prolonged transport, improper storage conditions, autolysis, antibiotic therapy, and other poorly defined toxic factors in samples may interfere with chlamydial isolation.

In this study, vaginal swabs proved to be excellent samples for detection of chlamydial infection in ewes with either the EIA or isolation procedures. They were easy to collect, readily available, and relatively clean. This EIA was a relatively sensitive and specific test for detection of chlamydial antigen in vaginal swabs. Swabs collected within 3 days after abortion were consistently strongly EIA positive and contained high numbers of viable chlamydiae. Chlamydial antigen was detected in vaginal discharges with this EIA for up to 42 days after abortion (average = 10 days). Additionally, chlamydiae were isolated from these secretions for up to 19 days after abortion (average = 8 days).

Additionally, this EIA readily detected chlamydial antigen in swabs of placental cotyledons; however, its specificity on these specimens was disappointing. There were relatively high numbers of true false positive results (8 of 65 results). Bacterial involvement was not apparent in these 8 placentas (i.e. no inflammation and no significant bacterial pathogens were isolated). However, these placentas were autolyzed and severely contaminated. Cross-reactions with gram-negative bacterial contaminants was the likely

cause of these false-positive results. This immunoassay has been shown to cross-react with several types of gram-negative bacteria including some strains of *Escherichia coli*.^{19,21,30,31} Cross-reactions may occur because this test utilizes an antibody that reacts with chlamydial lipopolysaccharide(LPS).^{21,31} Chlamydial LPS possesses at least 3 distinct antigenic domains, 2 of which are shared by other gram-negative bacteria and 1 that is unique to the genus *Chlamydia*.³² The antibody in this EIA may recognize these shared LPS antigenic determinants. The consistently negative EIA results from swabs collected from normal ewes suggested that the resident vaginal flora did not cross-react with this test. Vaginal swabs from 15 ewes with severe postpartum metritis due to infections with a variety of bacteria including *E. coli*, *Klebsiella sp.*, *Pseudomonas sp.*, *Actinomyces pyogenes*, *Ureaplasma sp.*, and streptococci, were EIA negative (personal observation). Nevertheless, false positive EIA results could occur when evaluating specimens from ewes aborting due to infections with other gram-negative bacteria.

This immunoassay was unable to consistently detect chlamydial antigen in swabs of other fetal tissues and body fluids. The amount of chlamydial antigen in these tissues probably was below the limits of detection of this immunoassay. When chlamydiae were isolated from these specimens they were present in low numbers. A comparative study demonstrated that this EIA was at least 100 X less likely to detect *C. psittaci* than isolation in cell culture.(A.A. Andersen, unpublished data) A swab specimen had to contain at least 300-400 chlamydial elementary bodies to give a moderately positive (OD 0.4-0.5) EIA result, whereas, isolation in cell culture could detect as few as 3-6 viable chlamydial

elementary bodies. With less sensitive isolation techniques, the difference between these two methods may be less.

Because of its cross-reactivity with other bacteria, this EIA is not suitable for routine use in a diagnostic laboratory setting where many specimens, particularly placentas, are often heavily contaminated. Additionally, this EIA could not consistently detect the small amount of chlamydial antigen in fetal tissues. Vaginal swabs proved to be excellent diagnostic specimens for chlamydial abortions in ewes. More sensitive and specific direct tests for detection of chlamydiae in ovine specimens are needed.

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7. EVALUATION OF A RAPID SOLID PHASE IMMUNOASSAY FOR DETECTION OF OVINE *CHLAMYDIA PSITTACI*

Summary

Kodak SureCell™ Chlamydia Test Kit, a commercially available solid-phase enzyme immunoassay (EIA) for the detection of *Chlamydia trachomatis* in human urogenital and conjunctival specimens was compared with isolation in cell culture for detection of *Chlamydia psittaci* in vaginal and placental swabs and swabs of fetal tissues from aborting ewes. This EIA had a sensitivity of 95.3% and 93.4% with placental and vaginal swabs from ewes experimentally infected with *C. psittaci* and field cases of abortion. On swabs of fetal tissues or body fluids from aborted fetuses or weak lambs from experimentally infected ewes this EIA had a sensitivity of 80.4% and a specificity of 94.5% compared with isolation in cell culture. Overall, this test had a sensitivity of 86.5% and specificity of 91.7%. This test proved useful for routine diagnosis of chlamydial abortions in sheep.

Introduction

Diagnostic tests designed specifically for direct (non-culture) detection of *Chlamydia psittaci* in animal specimens are not available commercially. However, several tests for *C. trachomatis*, particularly enzyme immunoassays (EIA) utilizing antibodies against chlamydial lipopolysaccharide (LPS), have been used for this purpose.^{2,4-9} Chlamydial LPS possesses at least 3 antigenic domains, 2 of which are shared by other gram-negative bacteria and 1 that is common only to members of the genus *Chlamydia*.¹ Generally, these tests have

proven to be less sensitive and specific than chlamydial isolation in cell culture. Cross-reactions with other gram-negative bacteria have limited the usefulness of some tests.⁵⁻⁸

Recently, direct-binding (solid-phase) enzyme immunoassays for detection of *C. trachomatis* have been introduced. These tests are self-contained, require no special equipment, and yield visual results. In this study, a commercial direct-binding monoclonal-antibody based enzyme immunoassay for the visual detection of chlamydial antigen in human endocervical, urethral, ocular, and urine specimens, was compared with isolation in cell culture for detection of *C. psittaci* in samples collected from aborting ewes. This test does not depend upon the viability of the organism. Chlamydial lipopolysaccharide (LPS) is extracted from the specimen and is detected with specific monoclonal antibodies.

Materials and Methods

Collection and Handling of Specimens

Vaginal swabs from ewes experimentally infected with C.psittaci: Placental and vaginal swabs were collected at abortion or lambing from twenty-three pregnant crossbred ewes that were inoculated intravenously with 1.0 ml of 0.01 M phosphate-buffered saline (PBS) (pH 7.2) containing approximately 10⁶ inclusion forming units of the ovine abortion strain B577 of *C. psittaci* in gestation days 85-110. Ewes were tested for chlamydial antibodies with a complement fixation test (National Veterinary Services Laboratories, Ames, IA) and results were negative. They were housed in isolation and observed twice daily for clinical signs of disease.

Two vaginal swabs were collected from each ewe at parturition. One swab was

immediately placed in a 12 x 75-mm polystyrene round-bottom tube containing 1 ml of Bovarnick's phosphate buffer (pH 7.4) and held at 4°C for up to 24 hours before isolation was attempted. The other swab was tested for chlamydial antigen with the EIA, and the results compared with isolation in cell culture.

Placental swabs from experimentally infected ewes and field cases of abortion: Swabs of placental cotyledons from experimentally infected ewes were tested for chlamydial antigen with the EIA. These cotyledons were also used for chlamydial isolation in cell culture in our laboratory.

Swabs from ovine placentas submitted to the Iowa Veterinary Diagnostic Laboratory, Ames, Iowa were also evaluated by EIA. Representative samples were collected and submitted to the National Veterinary Services Laboratories, Ames, Iowa, for chlamydial isolation in cell culture.

Swabs of fetal tissues and body fluids: Swabs of abomasal contents, lungs, liver, abdominal or thoracic fluids, and conjunctiva were collected from aborted fetuses and weak lambs from experimentally infected ewes. Samples from tissues were collected by vigorously swabbing the cut surfaces. They were placed in transport tubes (supplied with the EIA kit), stored at 4° C for 24-48 hours, and tested for chlamydial antigen by EIA. Representative samples of tissues/fluids were utilized for chlamydial isolation in cell culture and for routine aerobic and anaerobic bacteriologic isolation procedures.

Enzyme Immunoassay (EIA)

The EIA (Kodak SureCell™ Chlamydia Test Kit, Eastman Kodak Co., Clinical Products Division, Rochester, NY) was performed according to the manufacturer's instructions. All reagents including wash solutions were provided with the test kit. Briefly, a swab specimen is treated with 3 reagents, including proteolytic enzymes and hydrogen peroxide, to extract chlamydial LPS. This solution is filtered and transferred to one well of a Chlamydial Test Cell and allowed to drain. The Chlamydia Test Cell contains 3 wells (1-sample; 1-negative control; and 1 positive control well that contains bound inactivated chlamydial antigen) with filter membranes and absorbent pads. The membranes (wells) are washed twice and horseradish-peroxidase labelled monoclonal antibodies to chlamydial LPS are added and allowed to drain. At least 2 minutes after the fluid has drained through the membrane, each well is washed 3 times. A leuco dye solution is added and after 3 minutes the level of red/pink color formed is read and compared to the color formed in negative and positive control wells. If the color produced in the sample well is darker than that produced in the negative control well, the sample is considered to contain chlamydial antigen.

Chlamydial Isolation

Placental and fetal tissues and vaginal swabs were utilized for chlamydial isolation in Vero cell culture as previously described.⁶ Chlamydiae were identified with an indirect FA technique using a group-reactive mouse monoclonal chlamydial antibody and fluorescein-conjugated anti-mouse immunoglobulin G (IgG heavy and light chain specific) (Organon

Teknika, Malvern, PA.) at a dilution of 1:30.

Results

Sixty-one placental specimens (23 experimentally infected and 38 field cases) and 25 vaginal swabs were examined by cell culture and the EIA (Table 9). Compared with isolation in cell culture, the EIA had a sensitivity of 95.3% and specificity of 94.0%. Swabs from 4 placentas were strongly positive for chlamydial antigen by EIA, but chlamydiae isolation results were negative (Table 9). These placentas were characterized by extensive suppurative inflammation and advanced autolysis. Numerous 0.3-0.6µm coccoid organisms that were morphologically compatible with chlamydiae were identified in placental exudate and microscopic sections stained by the method of Gimenez. In addition, an immunohistochemical procedure utilizing mouse monoclonal antibodies to an ovine abortion isolate of *C. psittaci* demonstrated abundant chlamydial antigen in formalin-fixed paraffin-embedded sections of these placentas. Accordingly, these four results represented failures of isolation procedures rather than false positive EIA results. Adjusting for them, the sensitivity and specificity of this EIA on placental and vaginal swabs was 96.0% and 100%, respectively.

One hundred thirty fetal tissues or body fluids were tested with the EIA and isolation in cell culture (Table 9). Low numbers of chlamydiae were isolated from 46 specimens. Of these 46 positive specimens, 37 were EIA positive. Compared with isolation, the EIA had a sensitivity of 80.4% and a specificity of 94.0%.

Table 9. Comparison of isolation in cell culture (ISO) and a commercial solid-phase enzyme immunoassay(EIA) for detection of *Chlamydia psittaci* in ovine tissues

Specimens	Number of specimens			
	ISO -, EIA -,	ISO +, EIA +	ISO +, EIA -	ISO -, EIA +
Placentas, vaginal swabs ^a	39	41	2	4 ^b
Fetal tissues ^c	79	37	9	5
Combined	118	78	11	9 ^b

^a Samples from ewes experimentally infected with *C. psittaci* and field cases of abortion.

^b Advanced autolysis; severe inflammation; immunohistochemical identification of chlamydial antigen.

^c Fetuses from ewes experimentally infected with *C. psittaci*.

Discussion

Overall, the EIA correctly identified 82 of 93 positive samples and 118 of 123 negative specimens for a sensitivity of 88.1% and a specificity of 96.0%. Positive and negative predictive values were 94.0% and 91.5%, respectively.

These results showed that depending upon the type of sample, the EIA demonstrated a sensitivity 4-20% (overall 12%) lower than chlamydial isolation in cell culture. Since placentas and vaginal swabs collected from ewes aborting due to chlamydial infection contained relatively large amounts of antigen, the EIA was very efficient at detecting positive specimens. However, when evaluating swabs of fetal tissues, which usually contained considerably fewer chlamydial organisms (less antigen), its sensitivity was less.

Nevertheless, this test was superior to some other immunoassays for detection of chlamydial antigen in ovine fetal tissues.^{6,7} Some of these fetal tissues may have contained chlamydial antigen at levels below the analytical sensitivities of this test. Perhaps grinding fetal tissues and testing the supernatant/tissue fluids would have improved the sensitivity of this test. Accordingly, the fetal tissues that gave false positive EIA results may have contained non-viable chlamydiae.

This EIA was highly specific for detection of chlamydial antigen in these specimens. In contrast to other chlamydial EIAs,^{6,9} cross-reactions with LPS antigenic determinants that are common to chlamydiae and other gram-negative organisms was not a problem with this immunoassay. Negative test results were obtained in our laboratory with isolates of *Salmonella* spp., *Escherichia coli*, and several preparations of LPS, all of which were known to cross-react with other chlamydial EIA tests.^{7,8}

Except for the distraction of an occasional slow draining test well, this EIA was simple to perform. The colored endpoint was visually sharp and generally there was increasing color development with increasing levels of chlamydial antigen. The EIA used in this study required 21 minutes to complete; the time requirement has since been reduced to 9 minutes.

We found this solid-phase EIA to be useful for the routine diagnosis of chlamydial infections associated with ovine abortions. Overall, this EIA proved to be highly specific and relatively sensitive for detection of chlamydial antigen in placentas and vaginal swabs from aborting ewes. A positive EIA result indicated that chlamydial antigen was present. However, a negative EIA result did not rule out infection with chlamydiae, particularly with

fetal tissues. This EIA was less sensitive than chlamydiae isolation procedures, particularly for ovine fetal tissues. The slightly reduced sensitivity must be weighed against its advantages: speed, cost, and less stringent requirements for handling and transport of samples. Cross-reactions with other gram-negative bacteria was not identified.

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**8. AN INDIRECT MICROIMMUNOFLUORESCENCE TEST
FOR DETECTION OF
CHLAMYDIAE-SPECIFIC ANTIBODIES IN OVINE FETAL FLUIDS**

Summary

The objective of this study was to evaluate an indirect microimmunofluorescence test (IMIF) for detection of chlamydiae specific antibodies in serum and/or thoracic fluids from aborted ovine fetuses. Fluids from 142 ovine fetuses, including 64 fetuses from 40 ewes that were experimentally infected with ovine abortion strain OSP of *Chlamydia psittaci* at gestation days 85-110, 10 fetuses from normal ewes, and 68 fetuses selected from those received at the Iowa Veterinary Diagnostic Laboratory were evaluated. Fetuses from experimentally infected ewes were examined 4-60 days after inoculation. The IMIF results were compared with the results of isolation procedures for chlamydiae, complement fixation serology for chlamydiae, and concentrations of IgG immunoglobulins in fetal fluids. Chlamydiae-specific antibodies were detected by IMIF in 28 of 38 fetuses infected with *C. psittaci*. Fetuses infected with chlamydiae for more than 30 days consistently had elevated IgG immunoglobulin levels and IMIF titers $\geq 1:64$. The immunoglobulin levels and titer of chlamydiae-specific antibodies increased with maturity of the fetus and duration of infection. No chlamydial antibodies were detected with the complement fixation test. IMIF results were negative in fetal fluids from fetuses aborted due to other causes. These results suggest that the IMIF is a useful diagnostic test for detection of chlamydia-specific antibodies in

ovine fetal fluids or serum. The IMIF is rapid, inexpensive, and lends itself to routine use in the diagnostic laboratory.

Introduction

Chlamydial abortion, which is caused by infection with ovine strains of *Chlamydia psittaci*, is a major cause of fetal loss in sheep.^{1,23,39-41} This disease occurs worldwide and in many areas is the most commonly diagnosed cause of ovine abortion.^{1,10} Placental and fetal infections with this gram-negative bacteria may result in abortion or the birth of stillborn or weak lambs.^{19,21,39,40}

Ruminant fetuses can respond immunologically to a wide variety of antigens.^{12,17,22,25,26,34-36} This capability varies with the age of the fetus, the antigen, and the route of antigen exposure.^{30,35} Immunologic competence in the ovine fetus develops during a short period around mid-gestation.¹⁴ Fetal sheep challenged after day 79 of gestation produce both IgM and IgG1 immunoglobulins; synthesis of IgG lags IgM production by 6-14 days. As the fetus matures its ability to produce immunoglobulins with increased specificity is enhanced.^{30,35} Base-line IgG immunoglobulin levels in clinically normal unstimulated ovine fetuses range from 10 mg/dl at 77 days of gestation to 22 mg/dl at birth.³⁵

The presence of specific fetal antibodies to an infectious agent provides evidence for prenatal infection.^{4,22,25,34,35} Generally it is believed that ovine fetal immunoglobulins result from endogenous synthesis,^{30,35,36} however, transfer of maternal antibodies to the fetus can occur with severe placentitis when there has been fusion of maternal and fetal circulations.³³ Several studies have demonstrated that elevated IgG levels are present more commonly in

aborted than non-aborted fetuses and that elevated levels suggest an infectious cause of abortion.^{20,22,25} Serologic examination of fetal serum and body fluids has been used to assist in diagnosis of ovine abortions.^{6,11,17,18,22,27,34,35}

Ovine fetuses infected with *C. psittaci* may have elevated levels of IgG and antibodies specific for chlamydiae.^{5,17,35,38} Fetal chlamydial antibodies are not detected with the standard complement-fixation test (CFT) for chlamydiae, but have been identified with immunodiffusion, enzyme immunoassays (ELISA) and immunoperoxidase procedures.^{5,31,40}

The objective of this study was to evaluate an indirect microimmunofluorescence test (IMIF) for detection of chlamydial antibodies in serum and/or thoracic fluids from aborted ovine fetuses. The IMIF has been used for serologic studies of chlamydial infections in humans and for immunologic classification of chlamydiae.^{2,8,16,32,42-45}

Materials and Methods

Forty pregnant ewes that were seronegative to *C. psittaci* by complement fixation, received 10^6 inclusion-forming units of strain OSP of *C. psittaci*, an ovine abortion strain maintained at the National Animal Disease Center. Inoculations were made intravenously in days 85-110 of gestation. The inoculum was prepared from tissue culture harvest suspended in sucrose phosphate glutamate buffer.³⁷ Sixty-four fetuses were collected at selected times from 4-60 days post-inoculation. Ten fetuses from 6 normal ewes also were examined and served as controls. Sixty-eight ovine fetuses selected from those submitted to the Iowa

Veterinary Diagnostic Laboratory, Ames, Iowa during the 1990-1991 and 1991-1992 lambing seasons (December-April) also were included in this study. Each fetus was necropsied and appropriate tissues were collected for bacteriology, histopathology, determination of IgG concentrations in fetal fluids, serology and virology.

IgG Immunoglobulins

Heart blood or fluid from the thoracic cavity was collected in a tube, centrifuged, and the supernatant fluid was stored at -20° C until tested. This fluid was called "fetal fluids". Fetal fluids were tested for IgG concentrations using single radial immunodiffusion methods as previously described.^{13,17,24}

Serology

Indirect microimmunofluorescence test (IMIF): Serial 2-fold dilutions of the fetal fluids were tested for chlamydiae-specific antibodies by the indirect microimmunofluorescence (IMIF) test that was performed as previously described.^{2,42,43,45} Briefly, chlamydial antigen mixed with a normal yolk sac preparation was placed in 0.4 µl dots on standard acid-alcohol cleaned microscope slides and allowed to air dry for 30 minutes. The slides were fixed with acetone for 10 minutes. After air drying, 2 µl of fetal fluids at the proper dilution was added to each dot and slides were incubated for 30 minutes in a humidified chamber at 37° C. The slides then were carefully washed four times with PBS (pH 7.2), four times with distilled water, allowed to air dry, and were stained with fluorescein-conjugated rabbit anti-sheep immunoglobulin G (heavy- and light-chain-specific) (Zymed Laboratories, San Francisco, CA) containing 0.5% Evans blue, at a dilution of 1:30. Staining was done

by adding 4 µl of fluorescein-conjugated antibody to each dot and incubating and washing the slides as described above. Stained slides were examined with an epifluorescence microscope at x 400 to x 600 magnification.

Chlamydial antigen used in the IMIF test was from tissue culture harvest. Chlamydiae were suspended in 0.01 M phosphate buffered saline (PBS) (pH 7.2) containing 0.02% concentration of formalin. This suspension was mixed with normal 10% yolk sac preparation and PBS at a ratio of 1:1:3 and thoroughly mixed with a vortex mixer.⁴⁶ Additional PBS was added if samples contained too much yolk sac material for proper testing. Yolk sac suspension facilitates adhesion of chlamydiae to the microscopic slides.

Antigen was placed on the slide in a pattern of four rows containing seven dots each (Fig. 55). Dots were spaced at 5 mm to facilitate microscopic examination. The first column of four dots was routinely tested with a group reactive monoclonal antibody to evaluate the antigen preparation for adequate amounts of chlamydial elementary bodies. Each of the remaining six columns was used for testing four dilutions of fetal fluids.

Toxoplasmosis: Fetal fluids with concentrations of IgG greater than 20 mg/dl were tested for antibodies to *Toxoplasma gondii* with a latex agglutination test (Toxotest-MT, Eichen Chemical Co. LTD., TANABE USA INC., San Diego, CA). Titers $\geq 1:16$ were considered indicative of prenatal infection.

Complement fixation test (CFT): Fetal fluids with IgG levels greater than 20 mg/dl were evaluated for chlamydial antibodies with a complement fixation procedure (National Veterinary Services Laboratories, Ames, IA).

Histopathology

Fetal lung, liver, kidney, spleen, heart, brain, lymph nodes, adrenal and placenta were fixed in 10% neutral buffered formalin, prepared by standard methods, stained with hematoxylin-eosin, and examined with light microscopy.

Bacteriology

Stomach contents collected with a sterile needle and syringe, lung, liver, and placentas (when available) were cultured aerobically and anaerobically on 5% bovine blood agar and campylobacter agar (Skirrow campylobacter agar, Difco Laboratories, Detroit MI).

Bacterial isolates were identified by standard methods.⁹ Stomach contents were examined by darkfield microscopy. When placental inflammation was identified macroscopically, smears of placental cotyledons were stained by the Gimenez method and examined for chlamydial elementary bodies.¹⁵

Chlamydial isolation: Placental and fetal tissues were utilized for chlamydial isolation in Vero cell culture as previously described.² Chlamydiae were identified with an indirect FA technique using a group-reactive mouse monoclonal chlamydial antibody and fluorescein-conjugated anti-mouse immunoglobulin G (IgG heavy and light chain specific)(Organon Teknika, Malvern, PA) at a dilution of 1:30.

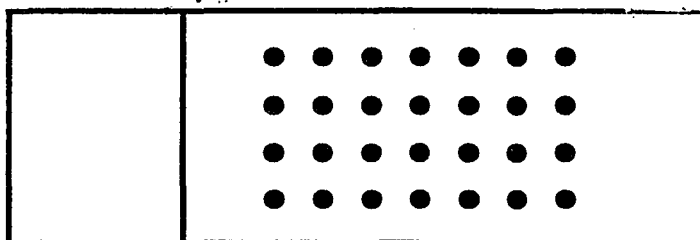
Virology

Fluorescent antibody tests: Spleen and kidney were examined for border disease virus with a direct fluorescent antibody procedure that utilized a fluorescein-labelled antibody to bovine virus diarrhea virus (Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA).

Virus isolation: Spleen and kidney were utilized for virus isolation procedures in bovine nasal turbinate cells (Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA).

Fig.55--Diagram of antigen pattern for IMIF test for chlamydiae-specific antibodies.

Antigen preparation is placed on a standard 25 X 75 mm glass microscopic slide as 0.4μl dots spaced at 5 mm intervals and in a pattern of 4 rows containing 7 dots.



Results

One hundred-forty-one ovine fetuses were evaluated in this study. The causes of these abortions are given in Table 10.

Fetuses from Ewes Experimentally Infected with C. psittaci

Chlamydial infection was identified in 38 of 64 fetuses from experimentally infected ewes. Infection was defined by isolation of *C. psittaci* in cell culture and/or demonstration of characteristic gross and microscopic placental lesions (i.e, necrotizing and suppurative placentitis with chlamydiae demonstrated in Gimenez-stained placental smears and/or microscopic sections). Mild placental inflammation was present in 14 fetuses examined 14-28 days post-inoculation (DPI). Moderate to severe suppurative placentitis was identified in 22 fetuses examined 30-60 DPI. High numbers of chlamydiae were isolated from all inflamed placentas. In addition, low numbers of chlamydiae were isolated from many fetal tissues. Microbiologic tests for other agents were negative.

Body fluids and serum from ovine fetuses infected with chlamydiae contained increased concentrations of IgG (mean=99 mg/dl) (Table 10). IgG levels tended to increase with duration of chlamydial infection. IgG levels in fluids from fetuses examined < 24 DPI were not increased (Table 2). Fluids from chlamydiae-infected fetuses examined 24-30 DPI contained increased levels of IgG (mean = 43 mg/dl; range 2-70 mg/dl). Markedly elevated levels of IgG were a consistent finding in fetuses examined 31-60 DPI (mean IgG=139 mg/dl, range 65-300 mg/dl) (Table 11). Fluids from several late term fetuses examined 50-60 DPI contained IgG levels of 250-300 mg/dl.

No abnormalities were identified in 10 fetuses collected from normal control ewes and in 26 other fetuses from experimentally infected ewes. All laboratory examinations were negative and the IgG concentrations in fetal fluids were less than 20 mg/dl (mean=12 mg/dl) in this group of 36 fetuses.

Field Cases of Abortion

Toxoplasmosis was diagnosed in 23 fetuses (Table 10). Characteristic microscopic lesions including multifocal placental necrosis and mineralization and/or multifocal necrotizing to granulomatous encephalitis were identified in 20 fetuses. Toxoplasma antibody titers $\geq 1:16$ were detected in fluids from 18 fetuses. Three fetuses did not have histologic evidence of toxoplasmosis, but antibody titers to the organism were detected in fetal fluids. IgG concentrations in fluids from fetuses infected with *T. gondii* were markedly increased (mean= 144 mg/dl, range 60-350 mg/dl) (Table 1). All other laboratory tests were negative.

Campylobacteriosis was diagnosed in 8 fetuses (Table 10). *C. jejuni* was isolated from various tissue from each fetus. Characteristic microscopic lesions including purulent placentitis, bronchopneumonia and multifocal necrotizing hepatitis were identified in 5 fetuses. Fetal fluids from 2 of 8 fetuses contained slightly increased concentrations of IgG (30 and 40 mg/dl).

Abortion due to infection with other bacteria including *Salmonella arizonae*, *Pasteurella* sp., *Bacteroides* sp., and *Actinomyces pyogenes* was diagnosed in 10 fetuses (Table 10). These organisms were isolated in relatively pure culture from multiple tissues and other

laboratory examinations were negative. Placentitis and/or purulent pneumonia were typical histologic changes. Fetal fluid IgG concentrations were less than 20 mg/dl (mean = 12 mg/dl, range 0-20 mg/dl).

Purulent placentitis and pneumonia, compatible with a bacterial infection, were identified in 3 fetuses without corresponding bacterial isolation. The level of IgG in fluids from one of these fetuses was slightly elevated (40 mg/dl).

Fetal death due to dystocia and/or maternal illness (i.e., pregnancy toxemia, vaginal prolapse, and failure of cervical dilation) was diagnosed in 6 cases (Table 10). All laboratory tests were negative and IgG concentrations in fetal fluids were less than 30 mg/dl (mean = 12 mg/dl, range 6-30 mg/dl).

Eighteen fetuses were diagnosed as idiopathic abortions. There were no significant gross or microscopic changes and microbiologic procedures were negative (Table 10).

Serology

IMIF: One hundred forty-two fetal fluid samples were evaluated for chlamydiae specific antibodies using the IMIF procedure. Chlamydiae-specific antibodies were detected in fluids from 28 of 38 fetuses infected with *C. psittaci* (Table 10). Non specific (background) staining often was a problem in dilutions less than 1:8, particularly in fluids from autolyzed fetuses. Consequently, in this study IMIF titers $\geq 1:8$ were considered positive results. No antibodies against chlamydiae were identified in fluids from fetuses examined less than 24 days after infection (Table 11). An IMIF titer of 1:8 was detected in fluids of one fetus examined 24 DPI. Fluids from 6 fetuses examined 25-32 DPI had IMIF

titers of 1/16 to 1/32 (Table 12). IMIF titers $\geq 1:64$ were detected in fluids from 21 of 22 fetuses examined 31-60 DPI (Table 12)(Figs. 56, 57). There was a positive relationship between duration of infection with chlamydiae, severity of placental inflammation, levels of IgG in fetal fluids, and the IMIF titer of chlamydiae-specific antibodies (Table 12).

Chlamydiae-specific antibodies were not identified in fetuses aborted due to other causes (Table 10).

CFT: Ewes inoculated with *C. psittaci* developed CFT titers of 1/40 to 1/320 by 21 DPI. Control ewes remained seronegative. Fetal fluids with IgG levels > 30 mg/dl were evaluated for antibodies to chlamydiae with the CFT and all were negative.

Table 10. Results of diagnostic procedures, IgG levels in fetal fluids (serum and/or thoracic fluids) and results of the indirect microimmunofluorescence (IMIF) test for antibodies to chlamydiae

Cause of Abortion	Number fetuses	IgG(mg/dl)		Number of fetuses with IMIF titer $\geq 1:8$
		mean	range	
<i>Chlamydia psittaci</i> ^a	38	99	0-300	28
<i>Toxoplasma gondii</i> ^a	23	144	60-350	0
<i>Pasteurella</i> sp. ^a	1	20		0
<i>Bacteroides</i> sp. ^a	2	12	9-15	0
<i>A. pyogenes</i> ^a	3	13	5-20	0
Infectious cause suspected ^c	3	28	15-40	0
Dystocia, illness	6	12	6-30	0
Idiopathic	18	13	0-35	0
Normal fetuses ^d	36	12	0-30	0

^a Confirmed by isolation of specific organisms and/or demonstration of characteristic gross and microscopic lesions.

^b Ovine fetuses from ewes experimentally infected with *C. psittaci* and examined 4-60 days post-inoculation.

^c Inflammation of placenta and/or fetal tissues; other laboratory procedures negative.

^d 10 fetuses from 6 uninfected control ewes; 26 fetuses from ewes experimentally infected with *C. psittaci* examined 4-47 days post-infection.

Table 11. Indirect microimmunofluorescence (IMIF) results and IgG levels in fluids (serum and/or thoracic fluids) of ovine fetuses infected with *C. psittaci* in relationship of number of days post-inoculation of the ewe

Number of fetuses in which chlamydial infection confirmed ^a	Days post-inoculation of ewes	IgG (mg/dl) in fetal fluids		Number of fetuses with IMIF \geq 1:8
		Mean	Range	
2	4-10	0		0
8	11-22	5	0-15	0
6	24-30	43	20-70	6
22	31-60	139	55-300	22

^a Chlamydial infection confirmed by demonstration of characteristic gross and microscopic lesions and/or isolation of *C. psittaci* in cell culture.

Table 12. Comparison of IgG levels with indirect microimmunofluorescence (IMIF) test titers in body fluids (serum and/or thoracic fluids) from ovine fetuses infected with *C. psittaci*

Number of fetuses in which chlamydial infection confirmed ^a	IMIF titer for chlamydial antibodies	IgG concentrations (mg/dl) in fetal body fluids:	
		Mean	Range
10	\leq 1:4	5	0-15
1	1:8	40	
4	1:16	45	25-50
2	1:32	60	40-70
5	1:64	71	40-130
2	1:128	115	90-140
5	1:256	140	80-200
9	\geq 1:512	183	110-300

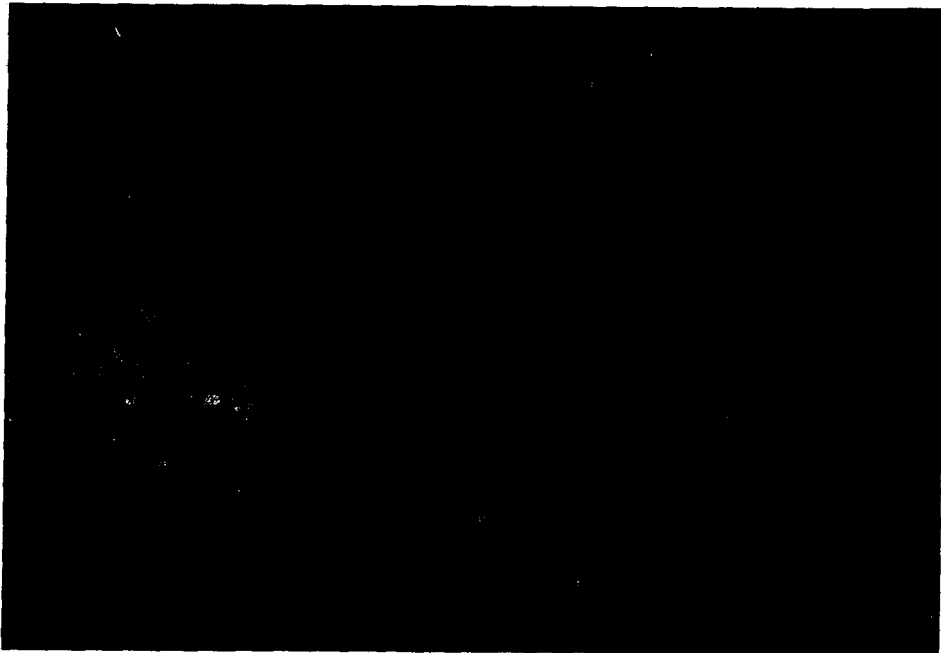
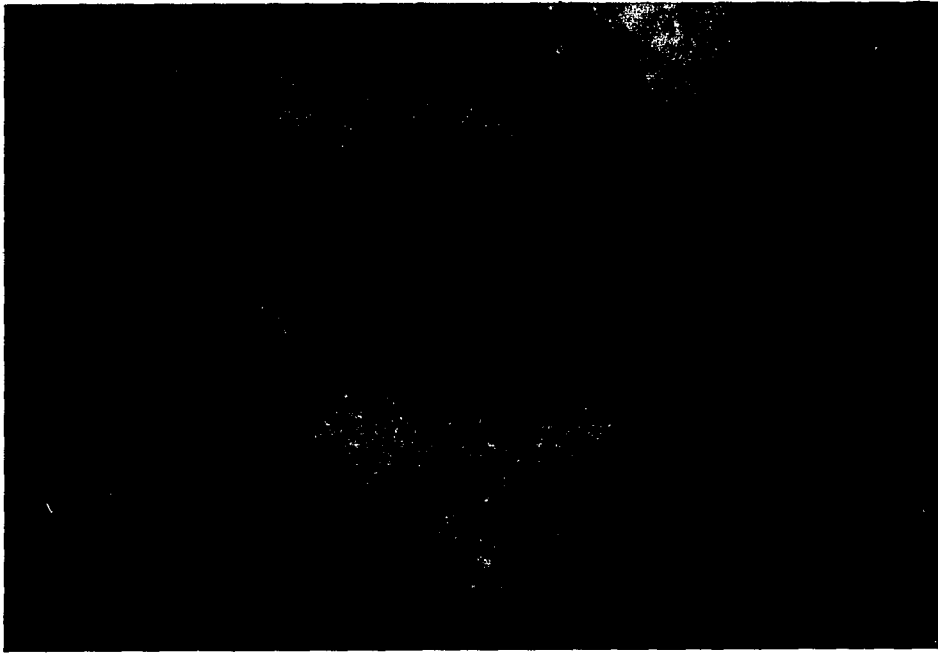
^a Chlamydial infection defined by isolation of *C. psittaci* and/or demonstration of characteristic gross and microscopic lesions.

Fig.56--IMIF evaluation of serum from an ovine fetus from a ewe inoculated intravenously with *C. psittaci* and examined 43 days post-inoculation. Note intense staining of chlamydial inclusions in egg yolk sac antigen preparations. Dilution of serum = 1:256.

Magnification = 400 x.

Fig. 57--IMIF evaluation of serum from an ovine fetus from a ewe inoculated intravenously with *C. psittaci* and examined 56 days post-inoculation. Note intense staining of chlamydial inclusions in egg yolk sac antigen preparations. Dilution of serum = 1:512.

Magnification = 400 x.



Discussion

The IMIF proved to be a useful test for detection of chlamydial antibodies in ovine fetal fluids. Chlamydiae-specific antibodies were first detected in a fetus examined 24 days after experimental inoculation. These results concurred with another recent study in which the immune response of ovine fetuses infected with *C. psittaci* were first detected with an immunoperoxidase method at 25 days post-infection.⁵ The immune response to chlamydiae increased with maturity of the fetus and duration of infection. Body fluids and sera from late-term ovine fetuses experimentally infected with chlamydiae for more than 30 days consistently contained chlamydiae-specific antibodies that were detected with the IMIF. IMIF titers $\geq 1:64$ were consistently detected in fluids of late-term chlamydiae-infected fetuses examined 30-60 DPI. The negative IMIF results from fetuses aborted due to other causes suggested that the IMIF was specific. Notable results were from fluids of fetuses aborted due to toxoplasmosis, which also contained high levels of IgG immunoglobulins, but were IMIF negative for chlamydiae specific antibodies.

Chlamydiae-specific antibodies were not detected in fetal fluids using the complement-fixation test (CFT). The CFT detects antibodies to lipopolysaccharide (LPS), the genus specific antigen present in all chlamydiae.^{3,7,28,29,39} In contrast, the IMIF which uses whole *C. psittaci* organisms, also should detect species- and strain-specific antigens. Previous studies have shown that the type of antigen influences the degree of the fetal immune response and the age at which fetal antibodies develop.^{30,35,36} Perhaps the immune response of fetal sheep to chlamydial LPS is weak or occurs late in gestation. Additionally, ovine fetal

antibodies may not fix complement. Other studies have demonstrated that the bovine fetus infected with *C. psittaci* responded with chlamydial antibodies that were reactive by immunodiffusion but did not fix complement.⁴¹

Body fluids and/or sera from ovine fetuses infected with either *C. psittaci* and *T. gondii* contained increased concentrations of IgG immunoglobulins. These fluids often contained more than 100 mg/dl of IgG. Accordingly, there was a positive relationship between increased concentrations of IgG immunoglobulins and the titers of chlamydiae-specific antibodies in ovine fetuses infected with chlamydiae. Infections with other bacteria usually did not stimulate significant increases in IgG immunoglobulins. These organisms may have killed the fetus before an immunologic response could be mounted.

The results of this study suggest that the IMIF test in conjunction with determinations of IgG levels in fetal thoracic fluids or serum are useful diagnostic methods for diagnosis of chlamydial abortions. When IgG immunoglobulin concentrations in fetal fluids are greater than 30 mg/dl, serologic examination for chlamydial with this IMIF procedure may be beneficial. IMIF titers of $\geq 1:8$ are indicative of prenatal infection with chlamydiae. However, the absence of specific fetal antibodies does not eliminate chlamydiae as the cause of an abortion. The IMIF is rapid, inexpensive, and lends itself to routine use in diagnostic laboratory. Additionally, the IMIF has proven useful for detection of chlamydiae specific antibodies in adult sheep.

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9. GENERAL DISCUSSION AND SUMMARY

Infection of susceptible ewes in gestation days 85-110 with abortion strains of *C. psittaci* resulted in placental infection with associated inflammation. The microscopic and ultrastructural findings indicated that erythrophagocytic trophoblasts lining placental hematomas were initially infected. Chlamydial infection of placental erythrophagocytic trophoblasts was first identified 14 days after inoculation. From the placental hematomas the infection rapidly spread to involve chorioallantoic trophoblasts of the peri- and interplacentome. The extent and severity of placental inflammation increased with the duration of infection. Moderate to severe suppurative placentitis with necrosis and vasculitis were consistent features of chlamydiae-infected placentas examined 30-60 days post-inoculation. High numbers of chlamydiae were isolated from infected placentas.

Chlamydial infection was limited to chorionic trophoblasts. Chlamydial replication resulted in degeneration and necrosis of chorionic trophoblasts which stimulated an intense neutrophilic inflammatory response. Seemingly, these inflammatory cells were ineffective at limiting chlamydial replication. Perhaps immaturity and/or features of the placental environment impairs the function of fetal leukocytes. The role of cell mediated immunity in placental resistance to microbial infection is poorly understood and warrants further investigation.

Ovine fetuses probably are infected hematogenously. In this study, chlamydiae were first isolated from fetuses examined 18 days after inoculation. However, isolation procedures were more successful in older fetuses examined 40-60 days post-inoculation. In

contrast to the placenta, fetal tissues contained low numbers of chlamydiae. There were no diagnostic macroscopic lesions in chlamydiae-infected fetuses. A variety of microscopic changes including lymphoid hyperplasia, infiltrates of mononuclear cells in portal areas of the liver, and multifocal and random hepatic necrosis were identified in fetuses infected with chlamydiae. Although not pathognomonic, these microscopic changes were suggestive of infection with chlamydiae.

Thoracic fluids and/or heart blood from chlamydiae-infected fetuses examined ≥ 24 days after inoculation consistently contained elevated levels of IgG and chlamydiae-specific antibodies that were detected with an indirect microimmunofluorescence procedure (IMIF).

IMIF titers $\geq 1:8$ were indicative of *in utero* infection with chlamydiae. There was a positive relationship between the duration of chlamydial infection, levels of IgG, and titer of chlamydiae-specific antibodies. IgG levels of 100-200 mg/dl and IMIF titers $\geq 1:64$ were common in chlamydiae-infected fetuses examined 40-60 days after inoculation.

Chlamydiae-specific antibodies were not identified in fetuses aborted due to other causes. The IMIF is rapid, relatively inexpensive, and well suited for routine use in a diagnostic laboratory.

An avidin-biotin-peroxidase complex (ABC) immunohistochemical method was developed and evaluated for detection of chlamydial antigen in formalin-fixed ovine tissues. Placentas infected with chlamydiae contained large amounts of chlamydial antigen that was readily identified with this ABC method. However, little or no chlamydial antigen was detected in other fetal tissues. It was likely that the amount of chlamydial antigen in many

fetal tissues was below the analytic limits of this immunohistochemical method. The inability of this test to consistently detect chlamydial antigen in fetal tissues greatly limits its usefulness. Future research should be directed towards development of other methods for identification of chlamydial antigen in formalin fixed ovine fetal tissues. Notable are the polymerase chain reaction (PCR) amplification methods. A PCR procedure has been developed for detection of chlamydial antigen in formalin fixed human tissues. Perhaps this procedure could be used for diagnosis of chlamydial infections in other species.

Other studies demonstrated that direct (non-culture) diagnostic tests designed to detect chlamydial infections in humans, were useful for identification of chlamydial infections in sheep. Two tests, Chlamydiazyme® a sandwich enzyme immunoassay and Kodak SureCell™ a solid phase immunoassay, were evaluated. Both tests were less sensitive than chlamydial isolation in cell culture. Cross-reactions with other gram-negative bacteria was a problem with the Chlamydiazyme® test and limited its usefulness. In contrast, cross-reactivity was not a problem with the solid phase immunoassay Kodak SureCell®. Swabs of placental cotyledons and/or vaginal exudate were the best specimens for detection of chlamydial antigen with these direct tests. Inconsistent results were obtained with swabs of fetal tissues. The reduced sensitivity of direct tests in comparison to isolation in cell culture must be weighed against their advantages: speed, cost, and less stringent requirements for handling and transport of samples. Based upon the results of these studies, it was concluded that because of its high specificity, the Kodak SureCell™ enzyme immunoassay was suitable for routine use in a veterinary diagnostic laboratory.

A goal of this research was to develop and evaluate other diagnostic methods for detection of chlamydial infections in sheep. If the placenta is available, diagnosis of chlamydial abortion usually is not difficult. The gross and microscopic changes in the placenta associated with chlamydial infection often are diagnostic. A diagnosis often can be confirmed by isolation of chlamydiae in tissue cell culture or embryonated eggs. If the placenta is severely autolyzed and unsuitable for isolation procedures and/or chlamydial isolation is not available, the ABC immunohistochemical procedure and/or solid phase enzyme immunoassays such as the Kodak SureCell™ are useful methods for confirming chlamydial infection in placental specimens.

Unfortunately, in the majority of ovine abortion cases submitted to the diagnostic laboratory, the placenta is not available. In these cases, confirming a diagnosis of chlamydial abortion can be challenging. Recommended diagnostic methods include: microscopic examination of major visceral organs (i.e., heart, lung, liver, brain, and spleen); chlamydial isolation procedures; determination of IgG levels in fetal thoracic fluids or heart blood; serologic examination of thoracic fluids or serum; and, evaluation for chlamydial antigen in tissue or conjunctival swabs with an enzyme immunoassay such as Kodak SureCell. As mentioned previously, a variety of microscopic changes may be observed in fetuses infected with chlamydiae, however, none are pathognomonic for infection with chlamydiae. Sections of spleen, lung, liver, lymph nodes and conjunctival swabs are the best specimens for chlamydial isolation procedures. Typically, fetal tissues contain low numbers of chlamydiae. Thoracic fluids and/or serum from all aborted fetuses should be evaluated for

IgG levels. Fluids with IgG levels > 30 mg/100 ml should be tested for chlamydiae-specific antibodies with the IMIF procedure. IMIF titers $\geq 1:8$ are indicative of *in utero* infection with chlamydiae. Additionally, swabs collected from the cut surface of the fetal liver can be tested for chlamydial antigen with an enzyme immunoassay such as the Kodak SureCell™. Although this immunoassay is less sensitive than isolation in cell culture, it is very specific and a positive test result provides strong evidence for infection with chlamydiae. With these diagnostic methods the diagnostician has a reasonable chance to confirm a chlamydial abortion.

Research needs to be directed towards development of more efficient chlamydial vaccines. Recently there has been a wealth of information regarding the immune response of sheep to chlamydiae, but much of this information is confusing and sometimes conflicting. Attention of investigators now is focused on the early events of chlamydiae-host cell interaction function in attachment, entry, and mechanisms for avoiding chlamydial destruction by lysosomes. Early events provide the best opportunities to abort chlamydial infection. Identification of chlamydial virulence factors and surface antigens that initiate infection also may be helpful for vaccine development.

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